A COMPUTER AID FOR CLINICAL HEMATOLOGY.  Reich, P.R. and Bleich, Howard*

A computer program was developed to assist medical students and physicians in diagnosing and treating hematological diseases. Based on the patient's blood counts it determines whether or not he has anemia, polycythemia, thrombocytopenia, thrombocytosis, leukopenia or leukocytosis. Defects in coagulation are also considered if the patient has a bleeding tendency. Questions concerning history, physical examination, and lab data related to his problem are asked and results analyzed by a branching logic network that checks each new datum for reasonableness and for internal consistency with previous information and then attempts to make a diagnosis whose confidence depends upon the information provided.

For example, megaloblastic anemia is considered as "possible" if the patient's red blood cells are macrocytic, but "certain" if megaloblasts as well are seen in the bone marrow. These diagnoses are then woven into a problem-oriented consultation note that includes a differential diagnosis, a discussion of the relevant pathophysiology, suggested additional diagnostic tests, and, depending upon the completeness of the requested input information, therapeutic suggestions. The evaluation note concludes with a list of references to the medical literature. The value of this program to students and physicians will be discussed and there will be a film demonstration of its use.

2.
MECHANISM OF ERYTHROPOIETIN (EP) PRODUCTION BY COBALT (COCl2). Miller, M.E.* Howard, D.* Stohlman, F., Jr., Planagan, P.* St. Elizabeth's Hospital, Boston, Mass. Increased production of EP in animals treated with COCl2 has been thought to result from renal "histotoxic" hypoxia; alterations in O2 affinity of Hgb. have not been considered. Rats were given one S.C. injection of 10, 20 or 25 um. of COCl2/100 g. Blood was sampled anaerobically; "in vivo" P50, pH, pCO2 and pO2 were measured. EP was assayed in plethoric CF1 mice. Data for 25 um. were:

<table>
<thead>
<tr>
<th>Hours</th>
<th>pH</th>
<th>pCO2 (mmHg)</th>
<th>pO2 (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.32 ± 0.0</td>
<td>46.5 ± 0.8</td>
<td>35.1 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>7.21 ± 0.03</td>
<td>47.3 ± 1.0</td>
<td>38.1 ± 0.7</td>
</tr>
<tr>
<td>6</td>
<td>7.35 ± 0.01</td>
<td>34.2 ± 0.7</td>
<td>32.7 ± 0.7</td>
</tr>
<tr>
<td>12</td>
<td>7.41 ± 0.1</td>
<td>29.1 ± 0.6</td>
<td>31.9 ± 0.4</td>
</tr>
<tr>
<td>24</td>
<td>7.35 ± 0.01</td>
<td>26.1 ± 0.5</td>
<td>33.3 ± 0.5</td>
</tr>
<tr>
<td>48</td>
<td>7.32 ± 0.01</td>
<td>35.9 ± 0.7</td>
<td>34.3 ± 0.6</td>
</tr>
</tbody>
</table>

Initially there was a dose related metabolic acidosis, accompanied at higher dose levels by a decrease in the O2 affinity of Hgb. Over the ensuing 3 hours a compensatory respiratory alkalosis developed with a dose related decrease in the pCO2, increase in pH and a subsequent increase in O2 affinity of Hgb., which resulted in a striking increase in production of EP at 12 hours. The differences in EP response at the various doses paralleled the degree of change in pH and its subsequent effect on the O2 affinity of Hgb. Higher doses led to a more profound initial acidosis with a greater compensatory respiratory alkalosis and hence a relatively greater change in O2 affinity of Hgb. and EP production. Thus, the erythroid response to COCl2 appears to be explained by changes in O2 delivery due to changes in the O2 dissociation curve rather than histotoxic hypoxia.

3.
PRIMATE TYPE-C VIRUS-RELATED REVERSE TRANSCRIPTASE FROM HUMAN ACUTE MYELOGENOUS LEUKEMIA (AML) CELLS. Gallagher, R.*, Mondal, H.*, Smith, R.C.*, Todaro, G.J.*, Lewis, R.J.*, and Gallo, R., C. Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, Maryland. Cytoplasmic "particles" from AML cells were demonstrated to synthesize DNA on endogenous 35S or 70S RNA templates in 8 of 9 cases studied. In two cases, the RNA-directed DNA polymerase catalyzing this reaction was isolated from other cellular DNA polymerases, not by conventional procedures for the purification of polymerase enzymes, but by successive purification of the cytoplasmic "particles" by a modification of previous procedures (Gallo et al., Proc. Nat. Acad. Sci., in press). Like mammalian type-C virus reverse transcriptase (R.T.), these AML enzymes preferred the synthetic oligomeric-homopolymeric hybrids oligo dT-poly rA and oligo dG-poly rC and had molecular weights of 70,000 daltons. Further, they were selectively inhibited by antisera to R.T. from primate type-C viruses. These results confirm those previously reported for R.DNA polymerase isolated from leukemic R.T. (Sarnagadharm et al., Nature New Biol. 240: 67, 1972; Todaro and Gallo, Nature 244: 206, 1973). In one case of AML, an additional enzyme was isolated with the characteristics of so-called "R-DNA" polymerase, which has previously been found in apparently virus-free mammalian cells. Like R.T., it catalyzed poly dT synthesis on oligo dT-poly rA, however, in contrast to R.T., it did not respond to oligo dG-poly rC and was not inhibited by primate type-C virus antipolymerase IgG. These results establish that primate (?) human)-specific R.T., which can be distinguished from all other known cellular DNA polymerases, is present in some and perhaps all AML cells.
SPECIFICITY OF HETEROANTISERA TO HUMAN LEUKEMIA ASSOCIATED ANTIGENS. Baker, H.A., Ramachandar, K.*, and Taub, P.N.,* Toronto Western Hospital, Toronto, Canada and Mount Sinai School of Medicine, New York, New York. We have previously reported (Nature 241: 93, 1973) that heteroantiserum to human leukemia blast cells, produced in mice previously rendered tolerant to remission leukocytes, showed specificity for the blast cells in a fluorescent antibody test. Eight such antisera have now been raised (6 vs. ALL cells, 2 vs. ALL cells), and tested for cytotoxicity against blast cells of 30 adults with acute leukemia (25 ALL, 5 AML), remission leukocytes from 8 of the leukemic patients (5 ALL, 3 AML), and peripheral blood leukocytes from 30 normal volunteers. Antisera raised against AML cells were cytotoxic in high titre to both myeloblasts and lymphoblasts but were not significantly cytotoxic to remission leukocytes or cells from normal volunteers. Antisera raised against ALL cells showed a higher titre to lymphoblasts than to myeloblasts, and virtually no cytotoxicity to remission cells or leukocytes from normal volunteers. Antisera to myeloblasts absorbed repeatedly with lymphoblasts retained significant cytotoxicity to the immunizing cell type, and vice versa. We conclude that leukemic myeloblasts and lymphoblasts share antigens that are not found on remission or normal leukocytes and also possess antigens that are specific for the morphological cell types.

5. PLASMA CELL ASYNCHRONY IN MYELOMA. Bernier, G.M. and Graham, R.C., Jr.*, Case Western Reserve University and University Hospitals, Cleveland, Ohio.

Whether a characteristic morphologic defect distinguishes myeloma cells from normal plasma cells is unresolved. Using preliminary gluteraldehyde-formaldehyde fixation with maximal preservation of nuclear chromatin patterns, we have examined the ultrastructural characteristics of bone marrow from 50 patients with monoclonal gammapathies of diverse etiology and 15 patients with reactive plasmacytosis. Virtually every marrow from patients with multiple myeloma and macroglobulinemia contained plasma cells with disparity between cytoplasmic development and nuclear maturation. The most marked degree of asynchrony was manifested by non-aggregated nuclear chromatin, prominent nucleoli, and dilated rough endoplasmic reticulum. Lesser degrees of plasma cell asynchrony were also seen in these marrows. In contrast, plasma cells of the patients with reactive plasmacytosis exhibited no asynchrony. When a plasmablast was observed in reactive marrows, nucleus and cytoplasm were equally primitive. The monoclonal gammapathy patients were graded according to clinical extent of disease (degree of anemia, azotemia, lytic lesions, amount of myeloma protein, depressed normal immunoglobulin) and the clinical grade so derived correlated closely with the degree of asynchrony. Patients with most advanced disease exhibited the most marked degree of asynchrony, while those with benign monoclonal gammapathy were normal by ultrastructural criteria. We conclude from these studies that asynchrony, a specific ultrastructural defect, characterizes the malignant monoclonal gammapathies, and that the degree of asynchrony is proportional to the clinical severity of the disease. Whether the degree of cellular asynchrony is of therapeutic or prognostic usefulness must be determined.

6. BIOCHEMICAL BASIS FOR MEMBRANE ALTERATIONS IN IRREVERSIBLY SICKLED CELLS (ISC). Lessin, L.S., and Walee, C.B., The George Washington University Medical Center, Washington, D.C. Membrane bound microaggregates in ISC's have recently been identified by electron microscopy (EM) in our laboratory. The present study was done to elucidate the biochemical basis for this membrane alteration which contributes to increased viscosity, cation leak and shortened survival of the ISC. Control and sickle (SS) cells were incubated for 24 hours in isotonie Krebs-Heinsch buffer with 100 mM glucose, pH 7.4, under 95% N2 or 5% O2 with 5% CO2. Cells were sampled at intervals for determination of % ISC's, ATP, membrane bound hemoglobin, and for freeze etch and transmission EM of whole cell and membrane preparations. ISC's generated from SS cells incubated under 95% N2 showed gradual increment for 12 hours with a sharp rise during the second 12 hours. ATP levels were maintained in SS and control cells for the entire incubation, but in SS cells fell after 16 hours, under deoxygenated conditions. Membrane bound hemoglobin measured by microbenzidine reaction in washed sonicated membranes of N2 incubated SS cells increased for the first 16 hours with rapid increment during the final 8 hours. Incubated control cells and oxygenated SS cells showed no appreciable binding of hemoglobin to the membrane. EM freeze-etch study of these preparations confirmed the presence of microaggregates bound to internal membrane surface increasing over 24-hours in correlation with increases in membrane bound hemoglobin and % ISC's. EM sections of macroaggregates showed diffuse dense membrane thickening up to 1000A by osmophilic material. These findings indicate that ISC's can be generated from SS cells by prolonged hypoxia. Membrane bound microaggregates appearing in ISC's correlate with membrane binding of hemoglobin in presence of adequate ATP. It appears, therefore, that the membrane lesion in ISC's is induced by membrane-hemoglobin interaction, independent of cellular ATP.
CLINICAL TRIALS OF THERAPY FOR SICKLE CELL VASO-OCCCLUSIVE CRISSES. Kraus, A.P., for the cooperative study group. University of Tennessee Medical Units, Memphis, TN. The following modes of intravenous therapy were used in a double blind controlled study: large doses of 15% urea in 10% invert sugar (Luis), smaller doses of 15% urea in 10% invert sugar (Muis), 10% urea in 10% invert sugar (Suis), NaHCO3 in 10% invert sugar (NBis), M/6 lactate (ML), 10% invert sugar (IS) and 5% dextrose in N/S saline (QS). Methods for patient selection, crisis definition and random selection of therapy were the same for each participating group. Therapeutic success failure was determined by blinded observers during the first 24 hrs. by group A, and during the first 48 hrs. by groups B and C. In study A, the response rate for 23 patients given Luis was similar to that for 27 given Is. Peak BUN for Luis patients was 173±36 mg%, with no difference in peak value or rate of rise between patients who did and did not respond. For study B the response rates were not statistically different for patients given Muis (n=28), Suis (n=14), NBis (n=37), or IS (n=38). Four of the Muis patients reached BUN values over 150 mg%. Three of these were treatment failures. In study C there was no difference in response to therapy with ML (n=20) and QS (n=23). Aside from diuresis in all patients (most marked in Luis and Muis), headache and vein irritation in a few receiving urine therapy were not significant. It is concluded that simple hydration with IS or QS is as good as Luis, Muis, Suis, NBis, or ML in the therapy of the acute, painful crisis of sickle cell anemia. However, the results are less than satisfactory for each treatment used.

8. DISSEMINATED INTRAVASCULAR COAGULATION IN EXPERIMENTAL GRAM-NEGATIVE SEPTICEMIA. Corrigan, J. J., Kienat, J.F.* and Pagel, C.J.* University of Arizona Medical Center, Tucson, Arizona. The beneficial effect of heparin therapy in humans with gram-negative septicemia and associated disseminated intravascular coagulation (DIC) is controversial. Although endotoxin treated animals have been used as the model for DIC recent evidence suggests that experimentally induced infection approximates more closely the human disease state. The purpose of this study was to develop a model for gram-negative septicemia and to investigate the coagulation mechanism in order to determine if and when DIC occurred. Rabbits were given either live Pasteurella multocida organism (1.8x10^9/Kg), heat killed organisms (2x10^9/Kg) or sterile culture broth (1.0 ml/kg) intraperitoneally. Serial blood cultures, plasma endotoxin levels (lysat test), platelet counts, and factors I, II, V and VIII levels were obtained by cardiac puncture. The results in 75 animals showed: animals given dead organisms or sterile broth had negative blood cultures and endotoxin levels and no change in the platelets or coagulation factors; rabbits given live organisms develop positive blood cultures and endotoxin levels at 2 hours which persisted to death, thrombocytopenia by 8 hours, and DIC (reduced factors I, II, V and VIII) after 8 hours. In all animals the presence of DIC correlated with the time of death. Early heparin therapy (1000 units/Kg, i.v.) in 20 animals did not improve or prolong survival rates. Mortality was 100% by 24 hours after the injection, with or without the anticoagulant. The data indicate that rabbits given P. multocida regularly develop gram-negative septicemia, endotoxemia, and coagulation changes compatible with DIC. The model can be used to investigate other hematological effects of gram-negative organisms in a setting which is similar to human disease.

9. HORMONALLY ALTERED THROMBOGENICITY IN RABBIT ILIAC ARTERIES. Gaynor, E. Montefiore Hospital and Medical Center and Albert Einstein College of Medicine, Bronx, New York. This study explores the hypothesis that vascular thrombotic response to injury is in part a function of the sex hormonal environment. Treatment with testosterone (Wolinsky, JCI 15:1,2552, 1972) or with estradiol (Fischer, Endocrinol. 91:1,227, 1972) results in profound biochemical alterations in the connective tissue of whole aortas in experimental rats. Since thrombogenicity has been shown to be a function of the type of connective tissue exposed in injured vessels, the effect of sex hormones on the interaction of platelet with subendothelium was studied. Depot IM injections of testosterone or estradiol were given weekly for 8-16 weeks. Right iliac arteries were selectively denuded of endothelium with an inflated balloon catheter introduced through the femoral artery. Rabbits were sacrificed by perfusion fixation of the aorta 10 minutes after blood flow in the injured iliac artery had been reestablished. Platelet interaction with exposed subendothelium was quantitated morphometrically in semi-thin cross sections of the vessel. The average per cent of the circumference having aggregates of more than 3-5 platelets was 4% (range, 1-10%) in five untreated controls, 9% (1-18%) in six estradiol treated rabbits, and 33% (15-57%) in six testosterone treated rabbits. By electron microscopy, a subendothelial preponderance of elastin-associated microfibrils was conspicuous in the estradiol treatment group, whereas testosterone treatment resulted in a dearth of these elements and relative predominance of amorphous vascular basement membrane. No collagen was seen at sites of thrombus formation. These data suggest that hormonally induced alterations in vascular connective tissue are morphologically reflected in the subendothelium, and are associated, in testosterone treated rabbits, with increased thrombogenicity.
10. CLINICAL ASPECTS OF PLATELET SIZE MEASUREMENTS IN THROMBOCYTOPENIA AND THROMBOCYTOTIC STATES. Zeigler, Z.R.*, Murphy, S., and Gardner, F.H. Presbyterian-University of Pennsylvania Medical Center, Philadelphia, Pennsylvania. Platelet diameters were measured from EDTA peripheral blood smears using an ocular micrometer and recorded as >3μ and >4μ. The normal values for these measurements (mean ± 2 S.D.) were 8.4 ± 8.1% and 1.1 ± 1.8%. Unlike Garg et al. (Ann. Int. Med. 77:361, 1972) who found increased megathrombocytes in a variety of non-thrombocytopenic patients, we found normal % large platelets in 11/11 non-thrombocytopenic patients with Systemic Lupus Erythematosus and in 10/10 patients with idiopathic Thrombocytopenic Purpura (ITP) in remission. In fact, normal size values were seen in 6 of 7 patients with ITP, intact spleens and platelet counts, 65,000-150,000/mm³. However, 6 of 9 patients with ITP post splenectomy had large platelets at counts of 60,000-120,000/mm³. In ITP, at any given platelet count between 60,000 and 120,000, platelet size was greater in the post splenectomy group. Platelet size was always increased in ITP with platelet counts <35,000/mm³. The conclusions drawn in the groups of patients described above were confirmed by electronic particle volume measurements. Microscopic platelet size was normal in 12/13 thrombocytopenic patients with chronic marrow hypoplasia. Therefore, size measurements reliably distinguished destructive thrombocytopenia from decreased production at platelet counts <35,000/mm³. Normal size values were seen in reactive thrombocytosis while 15/24 patients with myeloproliferative syndromes (MPS) had increased >4μ. We conclude that platelet size measurements cannot be used to detect the compensated thrombocytolytic state and that moderate thrombocytopenia must be present before large platelets are seen in ITP. However, platelet size measurements are useful in the differential diagnosis of thrombocytopenic and thrombocytotic states.

11. I. CELL METABOLISM

ROLE OF THE SPLEEN IN HEREDITARY STomatocytosis. Weneder, P. Oakland VA Hospital and the University of Pittsburgh, Pittsburgh, Pa. It has been suggested that acanthocytes from patients with abetalipoproteinemia have an excess of membrane relative to volume which contributes to the spiny cell shape. We have measured red cell surface area by three independent methods: critical hemolytic volume (JCI 5:113), phthalate ester method (Abstr. 186, this Soc. 1972), and lipid content. Red cell volume was measured by 311 elution and surface, and mental methods, and mean channel on a calibrated Coulter Channellyzer. Two affected brothers (Ped 48:91) were studied.

<table>
<thead>
<tr>
<th>Units</th>
<th>Normal R</th>
<th>R. #1</th>
<th>R. #2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (μm³)</td>
<td>100</td>
<td>106</td>
<td>96</td>
</tr>
<tr>
<td>Area (CHV)</td>
<td>196</td>
<td>200</td>
<td>184</td>
</tr>
<tr>
<td>Area (Phthalate)</td>
<td>194</td>
<td>212</td>
<td>183</td>
</tr>
<tr>
<td>Phospholipid/cell mg x 10^-10</td>
<td>2.95</td>
<td>2.54</td>
<td>2.40</td>
</tr>
<tr>
<td>Cholesterol/cell mg x 10^-10</td>
<td>1.45</td>
<td>1.50</td>
<td>1.46</td>
</tr>
</tbody>
</table>

The data indicates that the shape change in abetalipoproteinemia is not accompanied by detectable alteration in either red cell volume or surface area.

The deformability of the acanthocytes was measured by filtration through polycarbonate filters (Nucleopore, 2.8μ pore size). No alteration in deformability was present. Since this differs from results reported for two other patients (Brit. J. Hematol. 18:383), alteration in erythrocyte deformability may be related to factors other than the basic disease.
ENERGY OF HYDROPEROXIDES IN THE METABOLISM OF GLUTATHIONE IN RED CELLS.

Srivastava, Satish K., Awasthi*, Yogesh, C., and Beutler, Ernest. City of Hope Med. Ctr., Duarte, CA. Reagents such as acetaldehyde, phenylhydrazine, ascorbic acid, primaquine, hydrogen peroxide, methylphenylhydrazine carboxylate (azoester) and diazene-carbonyl acid bis (N,N-dimethylamine) (diamide) have been long used for the oxidation of RBC GSH. However, these reagents are relatively non-specific for the oxidation of GSH; they all indiscriminately oxidize sulfhydryls. We now introduce two new reagents, t-butyl hydroperoxide and cumene hydroperoxide. These substances act as substrates for glutathione peroxidase and specifically enzymatically oxidize GSH in the RBC. In the presence of equimolar concentrations of hydroperoxides with respect to GSH, the half-life of GSH in the intact RBC is about 5 seconds at 37°C. In the presence of glucose, GSH is completely regenerated in normal RBC in about 25 minutes, whereas glucose-6-P dehydrogenase deficient RBC fail to regenerate GSH. However, these reagents also do not affect the activity of various glycolytic and glucose shunt pathway enzymes. Using these reagents, we have confirmed the existence of active transport of GSSG from the RBC. Our results indicate that hydroperoxides are superior agents for the oxidation of RBC GSH.


We recently observed that D-glyceraldehyde was rapidly consumed when incubated with blood cells, and that some of the sugar was converted to glycerol. The conversion of glyceraldehyde to glycerol was accompanied by a nearly stoichiometric increase in the rate of oxidation of glucose to carbon dioxide, indicating that the reduction of D-glyceraldehyde to glycerol was an NADPH-linked reaction. Two mammalian enzymes are known to have the capacity to reduce glyceraldehyde to glycerol, aldose reductase (Alditol:NADP oxidoreductase, EC 1.1.1.1) and L-hexonate dehydrogenase (L-Gulonate:NADP oxidoreductase, EC 1.1.1.19). The glyceraldehyde-reducing enzyme was purified over 500-fold by chromatography on DEAE. Kinetic characterization of the hemoglobin-free enzyme in phosphate buffer, pH 7.2 revealed a capacity to reduce glucose with a Km of 396 mM; glucuronic acid with a Km of 1.91 mM; galactose with a Km of 90 mM, and xylose with two Km values, 9 and 470 mM. These characteristics, the relative Vmax, and the ionic strength required to elute the enzyme from DEAE clearly established that the glyceraldehyde-reducing enzyme in erythrocytes is L-hexonate dehydrogenase. This enzyme is undoubtedly responsible for the accumulation of sorbitol which has been reported to occur in erythrocytes in the presence of high concentrations of glucose and may represent the first step in the “high Km” pathway for galactose utilization.
16.

AN ESSENTIAL ROLE FOR PHOSPHOGLYCERATE KINASE DEPENDENT RED CELL CATION TRANSPORT. Segel, G.H.*; Feig, S.A.; Glader, B.E.; Müller, A.* and Dutcher, P.* University of Rochester School of Medicine and Dentistry, Rochester, New York and Children's Medical Center, Harvard Medical School, Boston, Mass. We have studied 1) normal, 2) phosphoglycerate kinase (PGK) deficient, and 3) glucose depleted (GD) red cells to clarify the relationship of Na-K transport to glycolysis. At normal internal sodium concentrations (Na\textsubscript{i}) ouabain induced Na\textsuperscript{+} accumulation and K\textsuperscript{-} loss are reduced in GD red cells in which triose phosphates are absent and 2,3-DPG is substrate for glycolysis. Na\textsuperscript{+} and K\textsuperscript{-} transport are normal in PK deficient red cells with 10 to 15% enzyme activity. At increased Na\textsubscript{i}, GD cells have a marked impairment of the increase in cation transport and ouabain inhibitable lactate and pyruvate production. These data indicate that minimal substrate flow through PGK in PK deficient red cells is sufficient to allow a normal response in Na-K transport and glycolysis at normal or elevated Na\textsubscript{i}. The small increase in Na-K transport and glycolysis in high Na\textsubscript{i} GD cells suggests that Na-K ATPase influences glycolysis by a minor PGK independent mechanism in addition to the major PGK dependent mechanism.

17.

METABOLIC RESPONSE OF RED CELLS TO INORGANIC PHOSPHATE AND PYRUVATE IN VITRO. Brain, M.C.; Lott, D.E.* and Card, R.T.* Department of Medicine, McMaster University, Hamilton, Canada. The incubation of washed human red cells in a bicarbonate buffer containing 0.5 to 5.0 mM inorganic phosphate (Pi) with stringent control of pH (7.4 ± 0.01) for 4 hours resulted in the accumulation of fructose-1,6-diphosphate and triose, the steady state levels of which were linearly proportional to the Pi conc\textsuperscript{0} in the media from 1.5 to 5.0 mM (r = 0.95), and no change in 2,3-diphosphoglycerate (DPG). The addition after 3 hours of pyruvate (0.2 mM) disturbed the equilibrium resulting in a fall in DPG and triose, and a rise in DPG. The addition of uniformly labelled \textsuperscript{14}C glucose to the incubation and the separation of glucose, FDP, DPG and lactate by ion-exchange chromatography after 15, 30 and 45 minutes and at the same intervals after the addition of pyruvate in 3 experiments demonstrated that the rise in DPG after pyruvate was due to reduced catabolism and not increased synthesis. Fluorometric assays of partially purified red cell pyruvate kinase (PK) in the presence of 15 \textmu M phosphoenolpyruvate with and without FDP demonstrated that the FDP conc\textsuperscript{0} causing half maximal stimulation of PK was 15 \textmu M, which corresponded to the steady state conc\textsuperscript{0} of FDP in the incubations. Thus, the accumulation of FDP in response to Pi (1.5 to 5.0 mM) in washed red cells can be explained by the activation of phosphofructokinase by Pi and the feed-forward regulation of PK by FDP. The findings confirm the importance of the regulatory balance of PFK and PK in controlling red cell metabolism and DPG conc\textsuperscript{0}, and suggest that the accumulation of FDP in washed red cells provides a sensitive index of the metabolic response of red cells to physiological changes in Pi conc\textsuperscript{0} and pH.

18.

ABNORMAL KINETICS OF POLYMORPHONUCLEAR LEUKOCYTE (PMN) DEGRANULATION IN CHRONIC GRANULOMATOUS DISEASE (CGD). Gold, S.B.*; Hanes, D.M.*; Stites, D.P.*; Ponce, B.*; and Fudenberg, H.H. University of California School of Medicine, San Francisco, California. The rate of degranulation of PMN isolated from 3 males with X-linked CGD and their mothers was studied. Degranulation was studied by measuring the kinetics of release of lysosomal enzymes into the medium when PMN were stimulated by a non-ingestible surface coated with aggregated IgG. The granules were exocytosed and the sequential release of 2 lysosomal enzymes, acid phosphatase and \textbeta-glucuronidase was measured. Quantities of enzymes liberated from stimulated and non-stimulated PMN were compared to total enzyme content as determined by triton lysis of whole cells. PMN from normal adults as well as adults and children with chronic bacterial infections served as controls. Total lysosomal enzyme content was normal in PMN from children with CGD and their mothers. However, lysosomal enzyme release was significantly impaired in both patients and their mothers after 5 min of "phagocytic" stimulation (\textbeta-glucuronidase, p = 0.02; acid phosphatase p < 0.005). By 15 min, the release of acid phosphatase remained significantly lower than normal (p = 0.01), while \textbeta-glucuronidase release approached normal levels. By 30 min there was no difference in quantities of lysosomal enzymes released from PMN of patients, their mothers and controls. This observation is consistent with that of others. Cell viability was confirmed by measurements of cytoplasmic lactic acid dehydrogenase and trypan blue exclusion. Kinetic analysis of lysosomal enzyme release using a non-ingestible phagocytic stimulus suggests that early degranulation is impaired in patients with X-linked CGD and in heterozygous carriers.
19.

COMPLEMENT MEDIATION OF NBT REDUCTION BY NEUTROPHILS DURING INFECTION

Davis, D.H.,* Siroty, W.C.,* Wekpler, B.B.* Cornell University Medical College
New York, N.Y. Serum factors governing Nitroblue Tetrazolium dye (NBT) reduction by polymorphonuclear leukocytes (PMN) have been studied in an experimental model of infection. Sterilely induced guinea pig PMN exhibit low levels of NBT reduction in the presence of normal serum. Serum activated with zymosan or heat killed bacteria stimulates NBT reduction by PMN in a nonphagocytic system. Inactivation of the serum complement by heat or EDTA led to markedly decreased NBT reduction, while EDTA treated serum permitted normal levels of NBT reduction suggesting that magnesium dependant alternate pathway activation is involved. Serum obtained from guinea pigs previously treated with cobra venom factor (CoF) to deplete C3-C9 showed significantly decreased NBT reduction. In vitro treatment with CoF to activate the complement increased NBT reduction. Serum taken from guinea pigs infected intraperitoneally with a virulent strain of Streptococcus stimulated NBT reduction by normal peritoneal PMN. This activity was destroyed by heat. Purified C3 briefly trypsinized to release C3a stimulated NBT reduction in a serum free nonphagocytic system. These results suggest that C3 activated by bacteria or their products during infection changes cellular metabolism resulting in increased NBT reduction by PMN in the absence of phagocytosis.

20.

EFFECTS OF ANEROBIASIS AND METABOLIC POISONS ON GRANULOCYTE O₂⁻ PRODUCTION. Curnutte, J.T.,* and Babior, B.M. New England Medical Center Hospital and Department of Medicine, Tufts University School of Medicine, Boston, Massachusetts.

We previously reported that human granulocytes produce superoxide (O₂⁻). More recently, we found that bacteria stimulate granulocyte O₂⁻ production by 2-4 x. Using resting (incubated without bacteria) or stimulated (incubated with bacteria) cells, we studied the effect of anaerobiosis and metabolic poisons on O₂⁻ production. A modification of the previously described assay (J Clin Invest 52:741, 1973) was used to detect O₂⁻. Resting or stimulated cells incubated under N₂ produced no O₂⁻. On introducing air, O₂⁻ production began, thereafter following the same time course as O₂⁻ production by cells incubated in air only. Poisons examined included Antimycin A, 2,4-dinitrophenol (DNP), N₃⁻, CN⁻, p-hydroxymercuribenzoate (pHMB), and F⁻. Control experiments showed no interference with O₂⁻ detection by these compounds. With resting and stimulated cells, little effect was seen with Antimycin A (3.3 µg/ml), DNP (0.1 mM), or N₃⁻ (0.1 mM). CN⁻ (1.0 mM) had no effect on O₂⁻ production during the first 10 minutes, although 20 minutes O₂⁻ production by stimulated cells had fallen somewhat below that of control. Inhibition was seen with pHMB (0.5 mM), resting O₂⁻ production falling to 4% of control. F⁻ (0.02 M) greatly increased O₂⁻ production. In the presence of F⁻, O₂⁻ production by resting and stimulated cells was equal, exceeding by 10 x O₂⁻ production by stimulated cells in the absence of F⁻. These results suggest that F⁻ activated by bacteria or their products during infection changes cellular metabolism resulting in increased NBT reduction by PMN in the absence of phagocytosis.

21.

BACTERICIDAL ACTIVITY OF HUMAN MONOCYTES. G.W. King*, S.P. Balcerzak, A.L. Sagone and A.P. Lobuglio. Department of Medicine, Ohio State University, Columbus, Ohio.

In animal models, activated macrophages have enhanced bactericidal and metabolic activity. This study examined the bactericidal activity of human monocytes (macrophage precursors) from normals and patients with tuberculosis (TB) and lymphoma. Mononuclear cells were isolated from venous blood by Ficoll-Hypaque centrifugation. Cell suspensions containing 2.5 x 10⁶ monocytes were then incubated with 12.5 x 10⁶ S. aureus in 1 ml Hank's solution with 0.1cc pooled AB serum. The number of viable bacteria were determined by colony counts at 0 time and after incubations of 30 and 60 minutes. The results are expressed in numbers of bacteria killed x 10⁶ (mean ± 1 S.D.):

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Normals (N=12)</th>
<th>Tuberculosis (N=15)</th>
<th>Lymphoma (N=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.5 ± 0.9</td>
<td>7.9 ± 2.7 (p &lt; 0.01)</td>
<td>5.7 ± 2.9 (p &gt; 0.1)</td>
</tr>
<tr>
<td>30</td>
<td>5.6 ± 1.2</td>
<td>8.0 ± 1.5 (p &lt; 0.001)</td>
<td>7.8 ± 2.4 (p &lt; 0.01)</td>
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</table>

Monocytes from TB patients had enhanced bactericidal activity consistent with macrophage activation induced by mycobacteria. Lymphoma patients also had monocyte activation with significant increments in cidal activity at 1 hour. Since granulocyte bactericidal activity is associated with stimulation of the hexose monophosphate (HMP) shunt, we examined glucose metabolism of pure monocyte monolayers in 6 male patients with lymphoma. They had significant increments in resting glucose utilization and ¹⁴CO₂ production from ¹⁴C-glucose (reflecting HMP shunt activity) when compared to normal male subjects. These results suggest that activated monocytes are present in patients with lymphoma and TB.
PATHOGENESIS OF ONE VARIANT OF SEA-BLUE HISTIOCYTOSIS. Golde, D.W., Schneider, E.L.*, Bainton, D.F., and Cline, M.J. Cancer Research Institute and Departments of Medicine and Pathology, University of California, San Francisco, California. The syndrome of the sea-blue histiocyte probably comprises a number of disorders of diverse etiologies. In none of these have the metabolic and hematologic pathogenetic mechanisms been clearly delineated. We studied 3 siblings of a family with documented sea-blue histiocytosis. Bone marrow cells were grown in vitro and skin fibroblasts were cultured and assayed for their content of enzymes involved in glycolipid metabolism. Specimens were also examined electron microscopically and histochemically. The bone marrow histiocytes were found to have the typical myelin lesions of Nieman-Pick disease. They also demonstrated histochemical reactions characteristic for sea-blue histiocytes. When the lipid in these cells was extracted with methanol-chloroform, the sea-blue material remained and was seen to be localized between the lipid vacuoles. Neutrophils ingested the sea-blue material liberated from degenerating macrophages in culture and were in turn phagocytized by other macrophages. Enzyme assays on fibroblast cultures showed the sphingomyelinase content to be 17.5% of control; glucocerebrosidase activity was normal. We conclude that partial sphingomyelinase deficiency can cause sea-blue histiocytosis and that the appearance of blue material is not due to methanol-chloroform-extractable lipid accumulation. Sea-blue histiocytosis is not a specific entity but may result from various metabolic abnormalities of the macrophage; one cause is partial deficiency of sphingomyelinase.

II. CHRONIC LEUKEMIA AND LYMPHOMA
CHRONIC GRANULOCYTIC LEUKEMIA (CGL) - CYCLING OF HEMOPOIETIC CELLS, ERYTHROPOIETIN AND COLONY STIMULATING FACTOR. Chikkappa, G.*, Borneo, G.*, Burlington, H.*, Cronkite, E.P., Pavlec, M.*, and Ohl, S.* Medical Dept., Brookhaven National Laboratory, Upton, N.Y. A striking oscillation of neutrophil (N), eosinophil, basophil, platelet, and reticulocyte (R) count was observed in an untreated patient with chronic granulocytic leukemia. The R count oscillated inversely with the neutrophil and other counts. The ratio of myeloblasts + promyelocytes to N myelocytes was 1:8.5 at the nadirs and 1:13.4 at the crests of the cycles suggesting a periodic expansion in the myelocytic compartment either due to an increased flow of stem cells into or additional mitoses within this compartment or both. The in vitro labeling of myelocytic DNA by 3H thymidine also oscillated in phase with the N count suggesting a reduction in the duration of the generative cycle (t<sub>c</sub>) implying the increasing N count is due to the reduction in t<sub>c</sub> with additional mitoses at the myelocyte level. Plasma levels of colony stimulating factor (CSF) cycled generally out of phase with N count suggesting that increases in CSF were responsible for the reduction in t<sub>c</sub>, potential additional mitoses and hence the increased rate of N production. Plasma erythropoietin (Ep) peaked nearly simultaneously with reticulocyte counts. Since Hgb levels were relatively constant the Ep oscillation appears due to enroachment on erythropoiesis by an expanding N mass, reducing utilization of Ep and thus allowing plasma Ep to increase. This in turn stimulates erythropoiesis producing a reticulocytosis. The overall picture suggests that there is competition for available stem cells. A cyclic production of leukocytic chalones has not been eliminated as a possible contributor to the N cycling. Further systematic study of hemopoiesis in similar cases of CGL may give insight into control mechanisms.

MARROW CHANGES IN CHRONIC GRANULOCYTIC LEUKEMIA AFTER TREATMENT WITH TOTAL-BODY IRRADIATION. Goswitz, P., Lushbaugh, C.* and Vodopick, H. Oak Ridge Associated Universities, Oak Ridge, Tennessee. We have compared marrow aspirates and/or biopsies three weeks postirradiation to preirradiated aspirates in 30 patients with chronic granulocytic leukemia who were given 50 treatments with total-body irradiation. Patients were treated in two special facilities designed to deliver a uniform field of gamma radiation to the entire body either at a low-exposure-rate of 1.5 R per hour (LETBI) or at a medium-exposure-rate of 1.5 R per minute (METBI). After exposures of 50R or less and 100R in LETBI, marrow showed no changes. After 100 R in METBI and 150R in both facilities, marrow cellularity often decreased and the number of megakaryocytes decreased slightly or moderately. The GE (granulocyte-erythroid) ratio fell after irradiation by an average of approximately 75% compared to the preirradiated ratio. In the majority of instances this reduced GE ratio at 3 weeks was proportional to the maximum percent drop in the blood leukocyte count from the preirradiated level. After exposures of 250R, the marrow cellularity, particularly of the erythroid precursors and of the megakaryocytes, decreased moderately. The most consistent change was a slight to moderate increase in fat. None of the marrows became normal in cellularity after a single treatment. For most patients, the marrow changes were less than anticipated, even in those who had more than a 90% drop after irradiation in the number of blood leukocytes. Almost all patients maintained an absence or a pronounced decrease of stored iron. In general, at the same total exposure, treatment with the higher dose-rate usually produced greater marrow changes.
ACID-RESISTANT VITAMIN B-12 BINDING CAPACITY (ARBC) AS A PARAMETER OF GRANULOCYTE TURNOVER. Gilbert, H.S. Mount Sinai School of Medicine of the City University of New York, New York.

Partial acid inhibition of vitamin B-12 uptake by serum was demonstrated by Miller and Sullivan (J. Lab. & Clin. Med. 53:607, 1959) who found that binders in human serum with one additional band were resistant to acid inhibition. Since an increase in serum content of R-type binders with acid mobility occurs in myeloproliferative and other disorders accompanied by increased acid inhibition, the value of ARBC in assessing bone marrow harvest was examined.

ARBC of sera from normals (19), patients with polycythemia vera (PV), chronic myelocytic leukemia (CML), secondary erythrocytosis (4), granulocytopenia (3) and leukemic reaction (1) was measured as $^{57}$Co-B-12 uptake ($pg/ml$) by serum at pH 1.5 (incubated at 37°C for 30”, with removal of free vitamin by Hg$^{2+}$-coated charcoal) and expressed as % of unsaturated vitamin B-12 binding capacity at neutral pH (UBBC). Normal ARBC was 369±177 or 30±9% of UBBC. Absolute values and ARBC % of UBBC were normal in secondary erythrocytosis (357±47, 37±5%), elevated in PV (1827±395, 67±13%), CML (3000±184, 89±10%), leukemoid reaction (3086, 92%) and reduced in granulocytopenia (37±24, 94%). Serial studies during myelosuppressive therapy showed a fall in ARBC as disease activity was controlled. Strong correlation (0.924) of ARBC with the amount of UBBC resistant to precipitation by half-saturation with (NH$_4$)$_2$SO$_4$ and the absence of acid-inhibited serum of a TC 11 peak on Sephadex G-200 gel filtration suggest that ARBC reflects predominantly the level of R-type serum binders. ARBC can be measured readily in conjunction with UBBC determinations and provides a simple screening method to assess granulocyte turnover and monitor disease activity and response to therapy without the need for column chromatography or gel filtration.

NONRANDOM CHROMOSOMAL ABNORMALITIES IN LEUKEMIA: Rowley, J.D.* (intr. by J.M. Baron) University of Chicago and the Franklin McLean Memorial Research Institute (operated by the University of Chicago for the United States Atomic Energy Commission), Chicago, Illinois.

Except for the Philadelphia (Ph$^+$) chromosome in chronic myelogenous leukemia (CML), the karyotypic patterns in patients with leukemia have appeared to be quite variable when the chromosomes were analyzed with the standard techniques. The chromosomal changes were thought to be merely an epiphenomenon. Re-examination of bone marrow chromosomes from these patients with the new techniques of quinacrine fluorescence and Giesma banding, has revealed that, in fact, chromosomal changes are nonrandom. In CML, cells from 21 consecutive Ph$^+$ positive patients showed a consistent abnormality in addition to the Ph$^+$ chromosome. With quinacrine fluorescence, an additional dull band was observed on the terminal portion of the long arm of one chromosome 9(9q+). Its appearance and size suggested that it was a translocation of the material missing from the Ph$^+$ chromosome. In the terminal phase of CML, nonrandom chromosomal changes occurred in five patients; one additional C chromosome in each of four patients resembled No. 8, and a metacentric marker chromosome noted in three patients contained at least one arm that resembled the long arm of No. 17. In acute myelogenous leukemia, an identical karyotypic pattern was noted in two of three female patients whose marrow cells showed a hypodiploid chromosomal number. Cells from both patients showed the loss of one X chromosome, as well as a translocation between chromosome 8 and 21 with breaks occurring in the same chromosomal band. It is proposed that these chromosomal changes reported herein may be the result of a different leukemogenic agent. Studies in animals, which indicate that specific nonrandom chromosomal changes are associated with different carcinogens, support this hypothesis.

STEM CELL KINETICS IN RPM MICE. Fried, W., Husseini, S., Trobaugh, P.E., Jr., and Knospe, U.H. University of Illinois Hospitals and Rush Presbyterian St. Luke’s Medical Center, Chicago, Illinois. RPM mice spontaneously develop a myelogenous leukemia which is transplantable into non-leukemic RPM mice. Leukemic cells have 39 chromosomes, whereas normal animals have 40. The kinetics of normal and leukemic stem cells were studied. Normal RPM mice were injected intravenously with $10^6$ spleen cells from leukemic ones. At weekly intervals thereafter until death (3-5 weeks) the following was done on batches of 10: total spleen weights, peripheral blood counts, nucleated cell counts, and colony forming units (CFU) in marrow, spleen and blood were assayed (CFU were assayed by counting the resulting macroscopic spleen colonies after injecting cells into lethally irradiated mice as per McCulloch and Till). The karyotypes and histologic appearance of cells in the resultant spleen colonies were studied. Narrow and spleen CFU increased 3-20 fold 2 or 3 weeks after injection of leukemic cells. One week after this, as the disease progressed, the number of marrow CFU dropped to normal, whereas splenic CFU dropped to a lesser extent and blood CFU remained high. The distribution of erythroid, granulocytic and megakaryocytic colonies derived from CFU assayed 1 week after injection of leukemic cells was normal. Two or 3 weeks afterwards most colonies were granulocytic. All spleen colonies from CFU assayed 1 week after injection of leukemic cells contained erythroid colonies with 40 chromosomes, whereas most from those obtained after 3 weeks had 39 chromosomes. The latter were all of granulocytic type. No cells in erythrocytic colonies contained 39 chromosomes. We conclude that the RPM leukemic stem cells differentiate only into the granulocytic series. Increase in the number of these cells inhibits first the non-leukemic CFU and later leukemic ones.
normal lymphocytes and the disease manifestation is suspected to be the result of lymphocyte biochemical abnormality with the biological consequences following DV, we have performed the UT-induced DNA repair in comparison with normal lymphocytes. In search for a correlate of this mitotic chromosomes, and 2) 95-100% cells are labelled after repair synthesis indicating that resistant (approximately 5 x) to this type of physical damage than their normal counterparts. DNA excisional repair exists uniformly in great majority of cells. After confirming that re-cell division after low doses of UV as evidenced by presence of 3H-thymidine label on the mitotic chromosomes, and 2) 95-100% cells are labelled after repair synthesis indicating that DNA excisional repair exists uniformly in great majority of cells. After confirming that retention of 3H-thymidine but not cell count and trypan blue exclusion represents surviving fraction of cells and by following the fate of 3H-radioactivity daily, a difference is demonstrated. The curve of decline of radioactivity in CLL cells after 100 ergs/mm² is similar to that of normal cells after 20 ergs/mm² of UV. We thus conclude that CLL lymphocytes are more resistant (approximately 5 x) to this type of physical damage than their normal counterparts. This finding seems to parallel the suspected longer life-span of CLL cells in vivo.


The presence of circulating lymphoma cells, vascular invasion by these cells and extranodal involvement indicates that blood borne metastasis does occur in lymphomas. An early step in metastasis formation is the attachment of the neoplastic cell to the vascular endothelium, but the mechanism and factors influencing this interaction are poorly understood. Thus, to further study this process, we developed an in vitro model system using a culture human lymphoma cell line, Raji, and cultured endothelial cells derived from human umbilical veins (SCIENCE 181, 453, 1973). After labelling with H-thymidine, 10⁵ washed Raji cells, in buffer, were added to confluent cultures of endothelial cells grown in 35 mm petri dishes. Each dish was then placed on a rocking platform. After 30 min of continuous rocking, the fluid was removed, and the monolayers were gently washed in buffer on the rocking platform for 5 min. After an additional wash, cells were either fixed in gluteraldehyde for microscopy or precipitated with trichloroacetic acid in order to determine the radioactivity of the attached Raji cells. This provided a quantitative measure of cellular adhesion. Microscopic and autoradiographic examination clearly demonstrated that Raji cells attached firmly to endothelial cells. This interaction was time, temperature and pH dependent suggesting the involvement of an enzyme system. Pretreating the Raji and endothelial cells with neuraminidase increased the degree of attachment only slightly suggesting that surface charge and the sialyl transferase system may not play a major role in this interaction. This model system should be helpful in studying the mechanisms and factors influencing the initial step necessary for blood borne metastasis to occur. In addition, agents which would inhibit this cell contact phenomena might be useful adjuvants in the prevention of vascular invasion and extranodal metastasis.
THE SPREAD OF HODGKIN’S DISEASE TO THE SPLEEN. Richard K. Desser,¹ Edgar M. Moran,¹ John E. Ulmmon, Donald J. Ferguson,¹ and Henry Rappaport. University of Chicago and Franklin McLean Memorial Research Institute, Chicago, Illinois 60637

The route of spread of Hodgkin’s disease (HD) to the spleen is unknown. Eighty-eight patients with untreated HD had a staging laparotomy that included splenectomy and biopsies of splenic, celiac, portal and bilateral para-aortic nodes as summarized below:

<table>
<thead>
<tr>
<th>Histology</th>
<th>Splenic + Nodes</th>
<th>Splenic + Splenic -</th>
<th>Splenic - Nodes +</th>
<th>Splenic - Nodes -</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytic Predominance (LP)</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Mixed Cellularity (MC)</td>
<td>25</td>
<td>9</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Lymphocytic Depletion (LD)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Nodular Sclerosis (NS)</td>
<td>55</td>
<td>18</td>
<td>0</td>
<td>4</td>
</tr>
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</table>

*Three patients had involvement of the spleen and splenic node only.

Splenic involvement was more common in LP (50%) and MC (61%) than in NS (33%). The spleen and abdominal nodes were positive simultaneously in 28 cases or negative simultaneously in 46 cases. Nodal involvement without splenic involvement (7 cases) was noted in 3 histologic types; splenic involvement without nodal involvement (7 cases) was documented only in LP and MC. Patients with intra-abdominal HD had more frequent involvement of the left supracavicular nodes (30/42) than did patients with no intra-abdominal disease (20/48) (p<.01). The frequent occurrence of isolated splenic HD in MC and LP suggests that hematogenous spread to the spleen may occur with these histologic types.

ARREST OF STAGE IV-B HODGKIN’S DISEASE FOLLOWING COMBINED BCG-TRANSFER FACTOR THERAPY. Moilvanie, S.K., Rockwood Clinic, Spokane, Washington. I have reported that BCG vaccinations at weekly intervals, followed by transfer factor (TF) will result in positive PPD reactions in cellular immune deficient patients (Lancet, July 21, 1973, p.148). Application of this approach is reported in the management of a 26-year-old man with MOPP-failure Stage IV Hodgkin’s disease (HD). Nodular sclerosing Stage III HD was diagnosed in 1968. Conventional cobalt therapy to 4,000 rads was delivered over upper mantle and the inverted Y-area including the spleen. Excellent resolution of tumors was followed in two months by relapse. His disease was then contained with bi-monthly vinblastine sulfate for 14 months. Four rib lesions occurred. The tentative conclusion is that he acquired cellular immunity with bi-monthly vinblastine sulfate for 14 months. Therefore, after 14 months MOPP therapy was stopped. Combined BCG-TF treatment was begun May 21, 1973. Freeze-thaw leukocyte lysate containing TF from 500 ml. of whole blood was used. This is considered equivalent to one unit of TF. The standard skin test antigens used were mumps, monilia, trichophyton and PPD. Retesting was done on the 14th post-TF day. Transfer factor from a PPD positive donor resulted in positive PPD, positive mumps and trichophyton skin tests. Four weekly injections of BCG then were given and PPD skin test retested weekly. All were neg. Four days after the second TF from a PPD-neg. donor all skin tests were positive, including a strongly reactive PPD. Subsequently, all signs of active HD disappeared, and calcification of the ribs occurred. The tentative conclusion is that he acquired cellular immunity following combined immunotherapy. This preliminary work will require further testing and refinement.

ACUTE LEUKEMIA COMPLICATING HODGKIN’S DISEASE IN REMISSION: ETIOLOGIC ROLE OF INTENSIVE THERAPY. Canellios, G.P., Whang-Peng, J., Arsenneau, J.C.*, Johnson, R.E.*, and DeVita, V.T. Medicine, Cell Biology, and Radiation Branches, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. In the last decade, improved survival of all stages of Hodgkin’s disease has resulted from the use of intensive radiation or chemotherapy. Prophylactic nodal irradiation without splenic involvement (7 cases) was noted in 3 histologic types; splenic involvement without nodal involvement (7 cases) was documented only in LP and MC. Patients with intra-abdominal HD had more frequent involvement of the left supracavicular nodes (30/42) than did patients with no intra-abdominal disease (20/48) (p<.01). The frequent occurrence of isolated splenic HD in MC and LP suggests that hematogenous spread to the spleen may occur with these histologic types.

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GLUTARALDEHYDE TREATMENT OF ERYTHROCYTES: A METHOD FOR INDUCING THEIR RAPID AND PREFERENTIAL CLEARANCE BY LIVER AND HEPATOMAS. Glew, R. H. *, Pinkard, G. D., * and Peters, S. P. *. Department of Biochemistry, University of Pittsburgh, School of Medicine, Pittsburgh, Pa. We have previously shown that a variety of enzymes and proteins can be entrapped inside erythrocytes by a procedure involving hypotonic shock (Proc. Nat. Acad. Sci., U.S.A., September, 1973). Our interest in developing procedures for loading red blood cells with enzymes as an approach for replacement therapy in lysosomal storage diseases has encouraged us to investigate methods and procedures for targeting such cells to specific organs, such as the liver, under conditions which preserve and optimize enzyme activity. In the present report we demonstrate using a 51Cr-labeling method that treatment of rabbit and rat erythrocytes with a solution of glutaraldehyde (0.25% in isotonic saline selectively (76%-81%) induces their rapid uptake by rabbit and rat livers. We have examined the effect of glutaraldehyde on some of the properties of soluble rat kidney β-glucosidase and β-galactosidase which were selected as representative of lysosomal hydrolases. Under these conditions of glutaraldehyde treatment, the following properties of these soluble glycosidases were not adversely affected: specific activity, Km, pH optimum and susceptibility to trypsin inactivation. Of considerable interest was the observation that preincubation of erythrocytes with glutaraldehyde encouraged experimental Morris rat hepatomas 7777 and 7800 to clear them at least as rapidly and selectively as the host livers. The possibility of treating hepatic tumors with glutaraldehyde-treated, drug-loaded red blood cells is discussed.

III. HEMOGLOBINS
CALCIIUM ACCUMULATION DURING SICKLING OF HEMOGLOBIN S (HbSS) RED CELLS. Palek, J.*., Center for Blood Research and Harvard Medical School, Boston, Mass. Membrane distortion during sickling is associated with increased transmembrane fluxes of K+ and Na+. We have studied whether this is accompanied by influx of Ca2+ into cells, which may further impair membrane properties of sickled erythrocytes. Calcium content of HbSS red cells determined by atomic absorption spectrophotometry (AAS) was 2.6 higher (P<0.01) than in control subjects and 1.7 times higher (P<0.02) than in other hemolytic anemias with similar reticulocyte counts. No correlation was found between red cell calcium and reticulocytosis. Study of 45Ca uptake during incubation of HbSS blood under nitrogen revealed (a) rapid initial 45Ca uptake (36 ± 29nM/1 cells/hour) which coincided with sickling and K+ efflux, (b) steady state after sickling was achieved and (c) further 45Ca uptake after ATP depletion. The calcium content of deoxygenated HbSS cells, determined by AAS, increased proportionately to 45Ca uptake, indicating a net gain in calcium. Upon repletion of normal cells which rapidly extruded Ca2+ accumulated during previous ATP depletion, no significant Ca2+ extrusion took place in reoxygenated HbSS cells despite normal ATP levels. The initial rapid Ca2+ uptake was not seen in HbSS cells incubated under oxygen or in red cells containing a high percentage of reticulocytes. It was abolished when sickling of deoxygenated HbSS cells was prevented by carbon monoxide. Calcium which accumulates during sickling may have deleterious effects on membranes of HbSS cells with diminished free ATP content.


We have developed a technique for the preparation of hybrid erythrocytes consisting of sickle cell membranes containing hemoglobin A or normal membranes containing hemoglobin S: Red cell ghosts are prepared by cold, hypotonic hemolysis and separated from their own hemoglobin by rapid centrifugation of the dilute hemolysate. Concentrated solutions of the desired replacement hemoglobin are equilibrated with the membrane preparations. The hybrids are then resealed by raising osmolarity and warming to 37°C. After washing, the reconstituted cells are assayed for cation retention and hemoglobin content, and the species of hemoglobin determined by electrophoresis. Average hemoglobin concentrations of 16gm/100ml, or about 50% of normal concentration, have been obtained in the tightly resealed cells. The residual native hemoglobin can be reduced to less than 1% of its initial concentration. Hybrids of normal cell membranes containing sickle hemoglobin sickle with metabisulfite or anoxia. The sickled hybrids are morphologically indistinguishable from natural sickle cells, though smaller (MCV 60±).

Such hybrid cells will be useful in studying membrane lipid instability, cation permeability, and cell desiccation or other membrane abnormalities which may be independent of the hemoglobin, even though they may be initially associated with the sickling process.
ALTERATIONS IN MEMBRANE FATTY ACID TRANSFER IN SICKLED ERYTHROCYTES. Lubin, E. Erythrocytes suspended in KHB buffer (pH 7.4, glucose 10 mM) were equilibrated at 37°C, PO₂ 100 mm Hg, for 20 min. The cells were then incubated at a PO₂ of 100 mm Hg or 20 mm Hg in the presence of palmitate-1-14C (PA) albumin complex. After 2 hr., the lipids were extracted and analyzed. Hypoxia induced major alterations in the relative incorporation of PA into phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) in the SS but not the controls (AA).

<table>
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<tr>
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<th>Percent Change with Hypoxia: PA as PC/PE, ± S.D.</th>
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<tbody>
<tr>
<td>SS</td>
<td>(+38.0)</td>
</tr>
<tr>
<td>AA</td>
<td>(-75.11)</td>
</tr>
<tr>
<td>SS</td>
<td>(+22)</td>
</tr>
<tr>
<td>SS</td>
<td>(+32)</td>
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Hypoxia had no influence on total RBC phospholipid or total incorporated PA. The increased PC/PE ratio seen in the hypoxic SS was due to increased accumulation of radioactivity in PC with a decrease in PE which was not dependent upon membrane fatty acid incorporation. It demonstrates an additional metabolic disturbance in the erythrocyte membrane induced by sickle hemoglobin.

SICKLE CELL DISEASE: DEMOGRAPHIC FACTORS INFLUENCING MORBIDITY. Powers, D., Haywood, L. J., Gilani, D. S., University of Southern California, Los Angeles, California. Ten consecutive years of clinical observation in 287 patients has been analyzed to determine significant demographic factors that affect the morbidity and mortality of patients with sickle cell disease (SS, SC, S thal). Distinct age-related features of disease manifestations were identified. More than 75% of the patients were under 15 years of age when first seen. Of the 37 who died (31 SS and 6 SC) 22 were less than 10 years of age. The modal age of death was 2 years. Frequency distribution patterns showed the highest morbidity and mortality in the first decade of life. Acute infectious diseases were the major cause of hospitalization and death in the childhood group. In the second decade, infectious morbidity decreased and illness was frequently related to adolescent and adjustment problems. Three patients died of thromboembolic phenomena, two of these patients were on birth control medication. During the third and subsequent decades, there was a random pattern of morbidity and mortality influenced by psycho-social problems and the cumulative deleterious effects on major organ function presumably from many years of uncontrolled sickling. None of the 12 deaths in the adult group were attributable to 'sickle cell crisis'. Morbidity of women was markedly increased as a result of pregnancy-related problems. Corresponding to the study by Serjeant in Jamaica, after the age of 30, hospitalized morbidity abated in both sexes. This study conclusively demonstrates that patients with sickle cell disease who are less than 10 years of age are at the highest risk with the majority of the problems being acute of onset, infectious in etiology and amenable to presently available treatment. Eighty seven per cent of the patients are alive. No definitive survival prognosis can be substantiated.

HEMOGLOBIN SYRACUSE (8143 His→Pro), A NEW VARIANT WITH HIGH OXYGEN AFFINITY DETECTED BY SPECIAL ELECTROPHORETIC METHODS. Jensen, M. I., Bunn, H. F., Nathan, D. G. and Oski, F. A. Dept. Pediatrics and Medicine, Children's Hosp. Med. Ctr. and Peter Bent Brigham Hosp., Boston, Mass. and Dept. Pediatrics, Upstate Med. Ctr., SUNY, Syracuse, N.Y. Family members from 4 generations were found to have polycythemia and increased whole blood O₂ affinity (P50: 11 mm Hg, normal: 27 mm Hg). No abnormal Hb bands were seen following electrophoresis on starch gel at pH 8.6 or agar gel at pH 6.0. Analysis of the oxygenated hemolysate by isoelectric focusing on polyacrylamide gel revealed 2 closely spaced bands. When deoxygenated hemolysate was analyzed on cell free gels, the two components were more widely separated. These alterations in membrane fatty acid incorporation demonstrate an additional metabolic disturbance in the erythrocyte membrane induced by sickle hemoglobin.
HEMOGLOBINS

HEMOGLOBIN ARLINGTON PARK (86 Glu-Lys, 95 Lys-Glu): AN ELECTROPHORETICALLY "SILENT" HEMOGLOBIN VARIANT WITH TWO AMINO ACID SUBSTITUTIONS IN THE SAME POLYPEPTIDE CHAIN. Adams, J.G.* and Heller, P. VA Westside Hospital and University of Illinois College of Medicine, Chicago, Illinois. Hemoglobin Arlington Park is indistinguishable from Hb A by cellulose acetate or starch gel electrophoresis at pH 8.6 or by acid agar electrophoresis at pH 6.2. It was discovered when the proband's hemolysate was chosen at random to serve as a normal control for peptide mapping. Separation of the component chain of this hemolysate on CM-cellulose columns by the method of Clegg et al. revealed a homogeneous α-chain peak which eluted in the position of Aα. Tryptic digests of this α-chain fraction contained all of the normal Aα peptides plus three additional peptides. Sequence analysis of these additional peptides and their normal counterparts demonstrated that in addition to Aα there was a variant β-chain present in which glutamic acid had been replaced by lysine at position 6 and lysine had been replaced by glutamic acid at position 95. The proband, a 46 year old black male, had no obvious hematologic abnormalities and died shortly after admission to the hospital of a subarachnoid hemorrhage. No other relatives of the proband have been located. Since Hb C (6 Glu-Lys) has been found in 2% of blacks in the United States and Mb N Baltimore (95 Lys-Glu) has been reported several times in the same population, Hb Arlington Park could have arisen from a homologous crossover in an individual doubly heterozygous for Hb C and Hb N Baltimore, or from a second mutation in a β-polypeptide chain which already carried the mutation for either Hb C or Hb N Baltimore.

THE MICRO MEASUREMENT OF FREE ERYTHROCYTE PORPHYRIN (FEP) AS A MEANS OF SCREENING FOR β-TALASSEMIA MINOR IN SUBJECTS WITH MICROCYTOSIS. Stockman, J.A.*, Weiner, L.B.*, Stuart, M.J.* and Oski, F.A. State University Hospital, State University of New York, Syracuse, New York. The micro measurement of free erythrocyte porphyrin (FEP) has proved to be a simple, rapid and reliable method for the screening of lead poisoning (Piomelli et al, Pediatrics, 51:254, 1973). Our experience indicates that it is also a simple and reliable method for rapidly distinguishing β-thalassemia minor from iron deficiency as a cause of microcytosis. Of 23 proven subjects with β-thalassemia minor, 22 had FEP values within the normal range (less than 67 ugms/100 ml RBC's). In one subject the value was 73 ugms/100 ml RBC's. In contrast, 93% of patients with iron deficiency in whom the MCV was 77 μ or less had elevations of their red cell FEP (mean 192 ugms/100 ml RBC's; range 43-700). All patients with iron binding saturations of less than 10%, regardless of red cell indices, had elevated FEP's; 70% of those with saturations ranging from 10-15% had elevated FEP's while no patient with an iron saturation of greater than 20% had an elevated FEP. The use of 0.01 ml of blood for the measurement of red cell FEP provides a rapid means of screening patients with microcytosis for the presence of β-thalassemia trait.

A NEW FORM OF β-TALASSEMIA TRAIT OF UNUSUAL SEVERITY. Friedman, S.*, Ozsoylu, S.*, Luddy, R.* and Schwartz, E. Children's Hospital of Philadelphia, Philadelphia, Pa. and the University of Maryland School of Medicine, Baltimore, Md. Beta thalassemia (thal) trait is usually a mild clinical disorder. We have studied 3 patients (including a father and son) of northern European origin who had anemia, marked reticulocytosis and normoblastemia, hypochromia and microcytosis. Splenectomy and cholecystectomy were necessary in the 2 adults, and the child had splenomegaly. Their Hb A2 and Hb F levels were elevated, in the range of β-thal trait. Bone marrow (BM) examinations showed marked erythroid hyperplasia, increased iron stores, and methyl violet inclusion bodies in red cell precursors. Peripheral blood (PB) and BM 14C-leucine labelled globin chains were separated by carboxymethyl cellulose (CMC) chromatography in BM urea. The PB ω/α ratios were 0.72, 0.78 and 0.80, intermediate between β-thal major and minor values. Hemolysates of BM were also studied by gel filtration chromatography. A peak of radioactivity eluting after the Hb tetramer peak was shown to consist of free ω chains. Radioactive ω chain in this pool was 37.7, 43.0 and 58.4% of the total radioactive ω chain, results similar to values previously found in β-thal minor. These 3 patients had reduced ω production in BM as well as in PB, thus differing from the usual heterozygote by a lack of ability to compensate fully in the BM for the ω-thal defect. This abnormality of globin synthesis, as well as the severity of the clinical features and the presence of numerous BM red cell inclusions, differentiates these patients from those with the typical form of β-thal trait and characterizes a new form of heterozygous β-thalassemia.
43. ABSENCE OF FUNCTIONAL B-GLOBIN mRNA IN HOMOZYGOUS B-THALASSEMIA. Kan, Y.W., Dorvy, A.M., and Holland, J.P.* San Francisco General Hospital, and University of California, San Francisco. Homozygous B-thalassemia can be divided into two types: (1) B+ in which a diminished amount of B-chain is produced, and (2) B- in which no B-chain is produced. The former is more common in patients of Mediterranean origin, and studies of mRNA by translation and DNA-RNA hybridization have shown decrease in the amount of functional B-mRNA. In contrast, in homozygous B-thalassemia from the Ferrara region, Conconi and his co-workers have reported stimulation of B-chain synthesis by addition of non-thalassemic supernatant factors in an endogenous cell-free system, and in vivo by blood transfusion, and have suggested that B-messenger is present. In the Oriental population, homozygous B-thalassemia is not uncommon. We have investigated the function of globin mRNA in two Chinese patients with homozygous B-thalassemia. Incubation of intact cells from the peripheral blood reticulocytes and from the bone marrow showed absence of B-chain synthesis. The mRNA was isolated from the peripheral blood reticulocytes of these patients and its ability to direct globin chain synthesis was assayed in a cell-free system derived from Krebs II ascites tumor. Even when globin synthesis was markedly stimulated by the addition of initiation factors from anemic rabbits, no B-chain synthesis was obtained. Hybridization of the patients' mRNA to radioactive DNA copied from 0-thalassemic mRNA showed reduced hybridizable B-mRNA. Thus we conclude that in homozygous B-thalassemia in the Chinese, functional B-globin mRNA is absent.

44. IMBALANCE IN A/B GLOBIN SYNTHESIS ASSOCIATED WITH A HEMOGLOBINOPATHY. Rieder, R.F., and James, G.M., III. S.U.N.Y. Downstate Med. Ctr., Brooklyn, N.Y. and Virginia Commonwealth University, Richmond, Va. In contrast to the thalassemia syndromes the structurally abnormal hemoglobins are generally associated with equal synthesis of α and β polypeptide chains. A study of globin biosynthesis in vitro in blood and marrow from two subjects heterozygous for unstable hemoglobin Leiden, B6 or 7 glu → α, revealed excess α chain production. A mother and daughter of northern European ancestry with mild microcytic anemia and compensated hemolysis were found to have 25 percent hemoglobin Leiden. Severe hemolysis occurred after ingestion of sulfonamides and during infections. Hemoglobin A2 content was 3.0 and 2.7 percent and hemoglobin F was 0.8 and 0.6 percent in the two subjects. Similar levels were found in unaffected family members. After incubation of peripheral blood with 3H-leucine for 10 minutes to 4 hours, the A/Leiden synthesis ratio was 1.3 and the specific activity of Leiden was 1.3-2 times A. These results indicate preferential destruction of unstable hemoglobin Leiden. The total A/α synthesis ratio was 0.47-0.63 in peripheral blood and 0.78 in marrow. A pool of free α chains was demonstrated by starch gel electrophoresis and DEAE column chromatography. The synthesis of globin chains was balanced in unaffected family members. This degree of predominance of α chain synthesis in subjects with hemoglobin Leiden resembles the findings in heterozygous B-thalassemia except that in cells with this abnormal globin production of both B and BLeiden appeared to be depressed relative to α. The Leiden deletion may therefore provide a model or structural analogue of the thalassemia syndromes, or an example of overproduction of α globin.

45. BALANCED GLOBIN CHAIN SYNTHESIS IN HEREDITARY PERSISTENCE OF FETAL HEMOGLOBIN (HPFH). Natta, C.L., Mazi, G., Ford, S., Marks, P.A., and Bank, A. Columbia U., Coll. of Phys. & Surg. New York, N.Y. Fetal hemoglobin (Hbf) synthesis compensates poorly for decreased α chain synthesis in α-thalassemia (α thal) major. By contrast, in patients with HPFH, total Hb production is normal due to adequate Hbf compensation. In the present study, globin chain synthesis has been quantitated in two Black families with HPFH. The results indicate that the HPFH gene is associated with balanced globin synthesis in reticulocytes. Two S-F patients also have balanced α chains. The propositus is completely balanced, the ratio being 0.9. His α/β synthesis ratio is 1.3. A sister is A-F with βa:816; Hbf:74.7%; HbA:23%; and heterogeneous Betke. Her α/β + γ synthesis is completely balanced, the ratio being 1.1. The second family has four A-F heterozygotes who are all clinically asymptomatic. They have higher Hbf levels (range 16-36%), homogeneous Betke's, and balanced α/β + γ synthesis. These data suggest that the asymptomatic clinical course in patients with HPFH is associated with balanced globin chain synthesis in reticulocytes.
46. GLOBIN SYNTHESIS IN FIBROBLASTS FUSED WITH ERYTHROBLASTS. Alter, B.P.* and Ingram, V.M.* (intr. by David G. Nathan) Dept. of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts.

The technique of cell fusion is useful for studies of gene regulation. Harris (Cell Fusion, Harvard Univ. Press, 1970) reported that hemoglobin synthesis was stimulated in erythroblasts fused with fibroblasts. In an attempt to activate the globin genes in fibroblasts, we have developed methods for the biochemical investigation of the quantity and type of globin produced in such fusions. Erythroblasts from 3 day chick embryos were fused with duck embryo fibroblasts with the aid of inactivated Sendai virus. The cells were incubated with radioactive amino acids and harvested at 24 hours after fusion, at which time an average of 14% of the cells were fused fibroblast-erythroblasts (heterokaryons). The soluble proteins were separated on SDS-urea polyacrylamide gels. The lysates from 12 fusion experiments showed a striking increase of radioactivity above background in the globin region. The fused cells produced on the average 6 times as much globin as did erythroblasts alone.

Analysis of the radioactive product on acid urea polyacrylamide gels showed that the fused cells definitely synthesized globin chains, which we are identifying further. These experiments confirm that fusion of erythroblasts with fibroblasts stimulates globin synthesis, and provide a method of identification of the globin chains.

47. IV. HEMOSTASIS AND THROMBOSIS I

REQUIREMENT OF IRON FOR PLATELET PROTEIN SYNTHESIS. Freedman, M.L., and Karpatkin, S. New York University Medical Center, N.Y. Previous work from our laboratory has shown that iron is required for maximum platelet production following acute or chronic blood loss, via the production of megathrombocytes (JCI 52:31, 1973). It was therefore postulated that iron was essential for the synthesis of an integral part of the platelet. This hypothesis was examined by testing the effect of an iron chelator as well as iron-containing compounds on platelet protein synthesis. Intact rabbit platelets were incubated at 37° for 1 hour in a Ringer's solution containing a mixture of radioactive amino acids. Following incubation, platelets were washed extensively and then treated with chloroform-methanol, hot TCA, ethanol-ether, and the remaining precipitate taken up in 0.5 N NaOH. This was analyzed for radioactivity and protein. Preliminary experiments revealed that protein synthesis was inhibited approximately 90% by either puromycin (200 ug/ml) or cycloheximide (10^{-2} M). 2,2'-bipyridine, a potent chelator of iron, reduced platelet protein synthesis by 80%. This could be reversed by addition of either transferrin at 5 ug/ml or hemin at 0.3 to 0.8 mM. Transferrin alone activated protein synthesis by 48% in 18 out of 28 experiments, whereas iron salts had no effect. Hemin alone activated protein synthesis in all experiments by as high as 170%. The hemin effect was different from the transferrin effect in that hemin activation was associated with platelet lysis and decreased platelet protein recovery. Never-the-less the specific activity obtained with hemin incubations was 2.7 fold greater than the specific activity obtained from water incubations in which comparable lysis was obtained. It is concluded that iron is required for platelet protein synthesis. Since iron (as hemin) is also required for hemoglobin synthesis, we suggest that it may be a universal requirement for mammalian protein synthesis.

48. GROWTH OF CULTURED ENDOTHELIAL CELLS ON VASCULAR GRAFTS. A.L. Annehberg*, L.J. Lewis* and J.C. Hoak. Department of Medicine, University of Iowa College of Medicine, Iowa City, Iowa.

The use of vascular prosthetic devices with artificial surfaces has been hindered by thrombotic complications. In part, they are related to the lack of a non-thrombogenic surface and delayed growth of endothelium on the surface. New methods developed in our laboratory now permit us to grow replicating human endothelial cells (EC), obtained from umbilical veins, in culture and use these cells in the study of endothelial growth on prosthetic devices. Vascular dacron grafts are not toxic to EC in culture. Cells have survived on fibers in culture for up to one and one-half months. Attempts to cover uncoated dacron devices with EC were not satisfactory. The cells would not bridge adjacent fibers. Precoating the devices with plasma clots gave a better surface to which the EC attached. However, EC produced a plasminogen activator and the plasma clot lysed over a period of 1-3 days. Experiments using I aminocaproic acid and platelet rich plasma in the clots were also performed to attempt to stabilize the clot surface. These cultures have been studied with light, transmission, and scanning electron microscopy. The results indicate that cultured cells did grow over a dacron graft covered with a plasma clot and that clot stability was essential for optimal growth. The role of platelets, erythrocytes and leukocytes is under study. This in vitro system provides a new approach for the study of this significant problem. If the use of tissue culture systems can avoid the immunological barrier associated with homologous transplants, this approach may have important clinical implications.
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49. LOSS OF PLATELET SURFACE PROTEIN DURING PLATELET-BLOOD VESSEL AND PLATELET-PHAGOCYTE CONTACT INTERACTIONS. George, J.J. and Sears, D.A. Department of Medicine, University of Texas Medical School at San Antonio. Morphologic studies of platelets have demonstrated a surface coat of material exterior to the plasma membrane, a potentially critical region in hemostatic contact interactions. We have developed a nonpenetrating label for platelet surface proteins, 125I-diazotized dinitrosulfanilic acid (I) (Clin Res 21:53 & 554, 1973) and have compared it to 51Cr with doubly-labeled platelets. Labeled platelets were washed 3 times, disrupted by sonication and fractionated on a sucrose gradient. 34-55% of I was present in the plasma membrane band and 34-54% of Cr in the soluble supernatant fraction. With further washing and in vitro incubation proportionately more of the soluble I was removed and a higher percent occurred in the membrane band. Labeled rabbit platelets recovered after 3 minutes in vitro circulation had lost all soluble surface I and some membrane I, with now all I in the membrane band. This correlated with the significantly more rapid exponential loss of I from doubly-labeled circulating rabbit platelets compared to the normal linear disappearance of Cr. More 1 than Cr of rabbit platelets remained attached to the tissues after in vitro incubation with fragments of autologous aorta (I: 2.20%, Cr: 1.04%, p < 0.002) and exposed endothelium of pulmonary arteries (I: 0.36%, Cr: 0.29%, p < 0.02) after removal of intact platelets by washing in saline and digitonin. More I than Cr of human platelets was phagocytosed by autologous granulocytes in the presence of antiserum (phagocytosis of I equivalent to 2.7 plt/WBC; phagocytosis of Cr equivalent to 0.48 plt/WBC, p < 0.05). We postulate that the I is labeling a peripheral zone of loosely-bound protein in addition to the plasma membrane, which may be important in platelet hemostasis and survival.

PULMONARY PLATELET THROMBOSIS AND SUDDEN DEATH INDUCED BY ARACHIDONIC ACID. M.J. Silver* and Hoch* Philadelphia, Pa. Arachidonic acid (AA) is known to cause irreversible aggregation of human platelets in vitro (Fed. Proc. 32, No.3, Absst. No.45, 1973). When the sodium salt of AA was injected into the ear veins of rabbits (dose 1.4 mg/kg contained in 1 ml and injected in 1 min.), the animals died within 1.5 to 3 min. after the injection. Sections of organs removed from rabbits immediately after death caused by AA revealed massive platelet thrombi in the small vessels of the lungs. The microcirculation of other organs was opened. This effect appears to be specific for AA. Closely related fatty acids (11,14,17 eicosatrienoic acid, 8,11, 14 eicosatrienonic acid, 5,8,11,14,17 eicosapentaenoic acid, docosahexaenoic acid, linoleic acid) did not cause death or gross toxic symptoms when injected at 3 times the lethal dose of AA. The fatty acid did not cause aggregation of AA, but all litter the vitro. If aspirin, an inhibitor of platelet aggregation, is administered prior to the injection of AA, the rabbits are protected from challenging injections of AA for several days. Indomethacin protects the animals for hours and sodium salicylate does not protect. These data indicate that AA can cause massive platelet aggregation, rapidly followed by blockade of the vessels of the microcirculation of the lungs by platelet thrombi leading to quick death by asphyxiation. AA is present in cholesterol esters, phospholipids and triglycerides in blood and tissues and, if suddenly freed, could cause local massive platelet aggregation. We propose that the sudden release of free arachidonic acid in circulating blood could be an important factor in pulmonary embolism and other disabling and fatal thrombotic diseases.

INHIBITION OF TISSUE FACTOR (THROMBOPLASTIN) ACTIVITY BY CONCANAVALIN-A: Zacharski, L.R., Phillips, P.G.* and Rosenstein, R.* VA Hospital, White River Jct., Vt., and Dartmouth Medical School, Hanover, N.H. Concanavalin-A (Con-A) blocks T.F. activity perhaps by binding to T.F. on the cell surface blocking such surface-related phenomena as cell migration, phagocytosis, and adhesion. Previous data demonstrating the superficiality of tissue factor (T.F.) on the plasma membrane and relating T.F. to adhesion and spreading of cultured fibroblasts (Nature 232:338, 1971; Blood 41:679, 1973) suggested that T.F. might be blocked by Con-A. Fibroblast monolayers possessing potent T.F. activity were washed x 2 with phosphate buffered saline (PBS) and then exposed to various concentrations of Con-A in PBS. Following 1 hour of incubation at room temperature the excess Con-A was removed by washing x 2 and PBS was added in a volume equivalent to that of the original culture medium. The cells were then mechanically dislodged, frozen and thawed to achieve lysis and then assayed with normal and factor VIII-deficient plasma. A progressive prolongation of the clotting time occurred which was proportional to the dose of Con-A. Maximum inhibition occurred within 1/2 hour of incubation. A similar inhibitory effect of Con-A was also observed on rabbit brain T.F. Addition of alpha methyl-D-mannopyranoside completely reversed the inhibitory effect of Con-A. When Con-A, in a concentration up to 10 x that calculated to be present on the monolayers at the time of assay, was added directly to test plasma or cell lysates without prior incubation, no prolongation of the clotting time was observed. These results indicate that Con-A blocks T.F. activity perhaps by binding to T.F. on the cell surface. In view of the known effects of Con-A on cell surface phenomena, it is hypothesized that T.F. may have physiologic significance related to these phenomena aside from its procoagulant effect.
CHARACTERIZATION OF PROSTAGLANDIN-CYCLIC AMP INTERACTIONS IN HUMAN BLOOD PLATELETS. Taylor, R. E.†, Stitt, E. S.; Robison, G. A.†, and Hartmann, R. C. Vanderbilt University School of Medicine, Nashville, TN. Recent studies have implicated a role for prostaglandin (PG) and the cyclic AMP (cAMP) system in the regulation of platelet function. We studied the functional and metabolic differences in the responses exhibited by human platelets after in vitro exposure to PGE1 and PGE2. Normal platelets were used as citrated platelet-rich plasma (PRP) or washed and resuspended in Eagles-glycylglycine buffer (PW). PW retained normal sensitivity to various aggregating agents, provided human fibrinogen was added. Platelets were incubated with PG (0.03-3 μM) and/or other agents at 37° with stirring (900 rpm). After acid precipitation, freeze-thawing, and purification on Dowex 50 columns, intracellular cAMP was determined by the competitive protein-binding method of Gilman. Preincubation with PGE1 inhibited ADP and epinephrine-induced aggregation, while PGE2 promoted aggregation. PGE1 produced a rapid increase (15 sec.) in platelet cAMP levels followed by a slow decline over 10 min. PGE2 produced a biphasic response, exhibiting a decrease in basal levels followed by a progressive increase over 5 min. The platelet cAMP response to PGE1 and PGE2 was significantly decreased or unchanged when PRP produced a biphasic response, exhibiting a decrease in basal levels followed by a progressive increase over 5 min. The platelet cAMP response to PGE1 and PGE2 was significantly decreased or unchanged when PRP or PW were preincubated with several antagonists (Li+, EGTA, phenolamine, SC1220, 7-oxo-13-prostaglandin). However, this antagonism to PGE1 and E2 was qualitatively and quantitatively different. The results of these studies suggest that the human platelet has separate receptor mechanisms for PGE1 and PGE2 leading to significantly different functional and biochemical responses. Likewise, the role of these two PG's appear quite diverse in regard to the regulation of platelet function.

EFFECT OF CYTOCHALASIN-D ON PLATELET FUNCTION. Puszkin, E.⁵ Puszkin, S.⁶ and Tanenbaum, S.⁷. (Intr. by Zalasky, R. J.). Mount Sinai School of Medicine of the City University of New York and Columbia University, New York, New York. The cytochalasins are a group of secondary fungal metabolites which are known to reversibly inhibit cell functions that are mediated by contractile proteins. The effect of Cytochalasin-D (CD) on human platelet function was studied. Exposure to low concentration of CD leads to ultrastructural changes in platelets with loss of granules and development of enlarged vacuoles. Metabolically, membrane and mitochondrial activity remain intact as measured by the uptake of 14C-acetate and 14C-glucose and their incorporation into the tricarboxylic acid cycle. CD had a slight effect on the primary wave of platelet aggregation by ADP and a normal secondary wave. CD per se, induced platelet aggregation and the release of 14C-serotonin, ADP and ATP. Aspirin blocked CD-induced aggregation. CD inhibited clot retraction and when added to platelet myosin, it inhibited the latter's ATPase activity and prevented myosin from associating with actin. Addition of CD to the actomyosin complex did not alter its ATPase activity. These data suggest that CD induces platelet aggregation by ADP release and interferes with clot retraction by interacting with myosin to a site required for actin binding, thus, inhibiting the formation of the actomyosin complex.

AN ACQUIRED BLEEDING DISORDER RESEMBLING THROMBASTHENIA DUE TO A PLASMA INHIBITOR. Kenoyer, G.°, Feinstein, D.I., Rapaport, S.I., Huey, F.° and Truax, V. Univ. So. Calif., L.A., Calif. A 55 year old woman developed large ecchymoses, severe epistaxis, and bleeding gums. A template bleeding time exceeded 5 minutes. Platelet count was 277,000/mm³; platelets appeared normal on the blood smear. F. VIII assay was 32%: other factor assays and prothrombin consumption test were normal. Her platelet-rich plasma did not aggregate with the following agents (final conc.): ADP (2 x 10⁻³), epinephrine (5.4 x 10⁻⁴⁴), collagen (3 x conc. aggregating normal platelet-rich plasma) -but did aggregate with thrombin (0.33 u/ml). A mixture of 1 part of her platelet-poor plasma plus 7 parts of normal platelet-rich plasma also failed to aggregate with ADP, epinephrine or collagen but aggregated with thrombin and Ristocetin® (1.5 mg/ml). She was treated with prednisone, 50 mg daily. Inhibitory activity decreased after 5 weeks and disappeared by 10 weeks. Bleeding time shortened to 51°; platelet counts were normal throughout. The inhibitor was present in serum and the plasma fraction precipitated by 50% (NH₄)₂SO₄. It was adsorbed onto bentonite (20 mg/ml), which did not remove measurable amounts of immunoglobulins. Heating her plasma or serum for 30° at 56°C decreased inhibitor activity by 50%. Adding 16 x 10⁹ fresh (< 72 h old) normal platelets to 1 ml of her plasma removed the inhibitor completely, whereas adding 2 x 10⁹ platelets partially removed the inhibitor. The inhibitor could be eluted from normal platelets with 2.5 M NaCl, 0.05 M tris buffer, pH 7.4 at 56°C for 30°. Attempts to neutralize activity with specific anti-IgG, IgA and IgM antisera or by affinity chromatography on anti-IgG-Sepharose were inconclusive because of dilutional loss of activity.
ACQUIRED "STORAGE POOL DISEASE" OF PLATELETS ASSOCIATED WITH CIRCULATING ANTI-PLATELET ANTIBODIES. Zahavi, J.* and Marder, V.J. Temple University Health Sciences Center and The Specialized Center of Research in Thrombosis, Philadelphia, Pennsylvania.

This study reports the first example of acquired "storage pool disease", in a patient with collagen vascular disease, thrombophlebitis and circulating anti-platelet antibodies. Studies of platelet function showed a long bleeding time, decreased aggregation to collagen, thrombin and high concentrations (>1μM) of adenosine diphosphate (ADP), and depleted stores of ADP and serotonin. These findings are similar to those present in a congenital disorder of platelets (Holmesen and Weise, Blood 39: 197, 1972) characterized by a deficiency of the storage pool of adenine nucleotides and serotonin. Relatively minor defects were also noted in primary aggregation with ADP at low concentrations (0.3-0.6 μM), platelet factor 3 activity induced by kaolin and clot retraction of whole blood and platelet rich plasma. These studies suggest that the platelet release reaction was induced by circulating antibodies, leading to depletion of the storage pools of adenine nucleotides and serotonin of circulating platelets. Thrombophlebitis might have originated from the precipitation of circulating platelet microaggregates induced by the circulating antibodies. The patient responded well to steroid therapy and platelet functions returned to normal concomitant with the disappearance of the anti-platelet antibodies. It is possible that "storage pool disease" with or without thrombosis may be operative in other acquired disorders, especially those associated with antibody formation.

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MECHANISM OF PLATELET DYSFUNCTION IN ACUTE LEUKEMIA. Cowan, D. H., Marsick, S. M.*, and Kolesar, J. D.* Case Western Reserve University School of Medicine, Cleveland, Ohio. Platelets from patients with acute leukemia are larger and younger than normal platelets and are functionally defective. To elucidate the mechanism underlying the impairment in function, the morphology, protein composition, and adenine nucleotide metabolism of "leukemic" platelets were assessed. As determined by SDS-acrylamide gel electrophoresis, the soluble fraction of sonicated platelets from 5 of 6 patients with acute leukemia lacked 3 or more proteins of M.W. 83,000 - 258,000 normally present and contained a large protein (M.W. 183,000) not normally present; the membrane fraction lacked one normal protein of M.W. 120,000 and contained an abnormal one with M.W. 238,000. The mean concentrations of ATP and ADP in resting platelets from 8 patients in relapse were significantly subnormal (p < 0.05, p < 0.01, respectively) and the decreases in platelet ATP and ADP after collagen stimulation were also significantly subnormal (p < 0.01 for each). The amounts of adenine nucleotides released were similarly reduced. The specific radioactivity of ATP in resting "leukemic" platelets after incubation with 3H-adenine was 0.5 x 1.1 x normal and that of ADP was 0.8 x 4.4 x normal. Degradation of ATP to IMP and hypoxanthine after collagen stimulation was 0 - 0.5 x normal in 4 patients and normal in the other 4. Reduced nucleotide release was associated morphologically with the presence of granules varying greatly in size and minimal granule migration and degranulation after exposure to collagen. The findings show that the qualitative protein composition of "leukemic" platelets is not normal. Additionally, abnormalities of structure and adenine nucleotide metabolism are present and contribute to the platelet dysfunction in leukemia.
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58.

ABNORMALITIES IN PLATELET FACTOR 3 AND GLUCOCEREBROSIDE CONTENT IN GAUCHER’S DISEASE. Swain, W.K.R., Desnick, R.J.*., Kaplan, M., and Krivit, W. V.A. Hospital and University of Minnesota School of Medicine, Minneapolis, Minnesota.

Platelet factor 3, availability (PF-3A) and content (PF-3C), is a platelet phospholipid-protein procoagulant. In an adult patient with Gaucher’s disease having easy bruising and mucous membrane bleeding since childhood, abnormalities of PF-3A and PF-3C were detected. The possible relationship of a PF-3 abnormality (modified method of Rabiner, S.F. and Hrodek, O.J. C. 47:791, 1968) and abnormal lipid storage characteristic of Gaucher’s disease prompted a study of WBC and plasma g-glucosidase (g-glu) levels, characteristically low in Gaucher’s disease; and glucocerebroside (GL-1) content, not previously measured in Gaucher’s disease platelets. Twelve family members were studied. Three, the propositus and 2 siblings, exhibited thrombocytopenia, grossly abnormal PF-3A and PF-3C, and white cell and plasma g-glu levels. The propositus was homozygous and the siblings were heterozygous by g-glu analysis. The parents and 2 siblings had normal platelet counts, moderately abnormal PF-3A, normal PF-3C and intermediate g-glu levels consistent with the heterozygous state. The other 5 family members had normal platelet counts, normal PF-3A and PF-3C. Enzyme levels were measured in only one and it was an intermediate value. Platelet GL-1 content of the propositus was 4 times normal. In an in vitro test system, the addition of GL-1 to 5 times normal levels markedly inhibited PF-3A. No platelet aggregation abnormalities were detected in the propositus. This is the first report of PF-3A and PF-3C abnormalities in Gaucher’s disease and these correlate well with WBC and plasma g-glu levels. Additional studies of PF-3 in Gaucher’s disease as it relates to g-glu levels and glucocerebroside storage are required.

59.

V. ANEMIA I

OXYGEN BINDING TO HEMOGLOBIN IN HYPOPROLIFERATIVE ANEMIA: ADAPTATION TO ALKALOSIS NOT HEMOGLOBIN DEFICIT. Marshall A. Lichtman, University of Rochester Medical Center, Rochester, NY

The relationships of hemoglobin (hb) concentration and blood pH to red cell 2,3-diphosphoglycerate (2,3-DPG) content and oxygen binding by hemoglobin have been studied in healthy subjects and subjects with reticulocytopenic anemia with or without severe chronic renal disease. In anemic subjects without renal disease, blood pH was inversely correlated with hb concentration (r = -0.70, P < .001) such that pH rose 0.05 unit for each 5.0 g/l decrement in hb; and red cell 2,3-DPG was also significantly correlated with blood pH (r = -0.71, P < .001) such that 2,3-DPG increased 3.2 [umoles/g hb for each 0.05 unit increase in pH. In subjects with chronic renal disease receiving regular hemodialytic treatment, predialysis pH was not increased with anemia and red cell 2,3-DPG was not significantly elevated. If daily oral NaCO3 was given, sustained alkalosis could be induced and red cell 2,3-DPG and P50 increased strikingly.

When all subjects were considered, the increment in pH with decrement in hb could explain 80% of the increase in P50 which occurred with anemia by the effect of a) hb concentration on pH, b) pH on red cell 2,3-DPG, and c) 2,3-DPG on P50. Importantly, P50 at the pH and base excess present in vivo was similar in all subjects, healthy, anemic and chronic renal disease with anemia whether an increase in 2,3-DPG occurred or not. Blood alkalosis and the accumulation of 2,3-DPG cancelled one another’s effect on red cell pH and thereby oxygen binding by hemoglobin. Hence, increased red cell 2,3-DPG and P50 compensated for the alkalosis of hypoproliferative anemia, not for the deficit in hemoglobin concentration.

60.

DECREASED ERYTHROPOIETIN SYNTHESIS AND INEFFECTIVE ERYTHROPOIESIS IN ACUTELY LEAD-POISONED RATS. Landaw, S.A. & Schooley, J.C.* V.A. Hospital, Syracuse, New York and Donner Laboratory, University of California, Berkeley.

The anemia of lead poisoning has been attributed to hemolysis and defective heme synthesis, although recent reports suggest a defect in RBC production and/or responsiveness to erythropoietin (ESF). Production of ESF was studied in rats receiving lead (40 mg/kg IV) immediately after a brief hypoxic exposure (2 hr @ 22,000 feet). The marked rise in serum ESF activity seen 2 hours after completion of hypoxia in the controls was significantly suppressed (p < 0.001) by the lead injection, indicating interference with de novo ESF synthesis. Heme turnover studies, using 14CO production, indicated a significant degree of ineffective erythropoiesis when lead was given at the same time as, or up to 22 hours after, injection of glycine-2-14C. It was not seen when lead was given 5 or more hours before tracer injection, or during the course of chronic lead intoxication. The effect was maximal when lead and tracer were given simultaneously (% of cohort destroyed), and decreased exponentially as the time interval between tracer and lead injection was increased. Two days after lead injection, reticulocyte percentage and incorporation of glycine-2-14C into circulating RBC were decreased to approximately 30% of normal (p < 0.05). These studies are further confirmation of the work of Norse et al (Blood.39:713,1972), and indicate that the erythroid hypoplasia induced after lead injection is the result of a decreased production of the trophic hormone (ESF) combined with intramedullary destruction of RBC precursors, most likely at the stage of the early normoblast. This effect seems to require the presence of transiently high blood lead levels, and may be the cause of occasional cases of erythroid aplasia occurring in lead poisoning in man.
ATP DEPENDENT PHOSPHORYLATION OF A MEMBRANE PROTEIN IN NORMAL AND HEREDITARY SPHEROCYTOSIS RED CELLS. Alfred Greenguist* and Stephen B. Shohet, Depts. of Medicine and Clinical Pathology, University of California, San Francisco. The kinetic and equilibrium properties of the phosphorylation and dephosphorylation of the spectrin component of human erythrocyte membranes were studied. Spectrin, which appears as a doublet component in sodium dodecyl-sulfate polyacrylamide gel electrophoresis, incorporated equivalent amounts of 32P label into each component upon incubation with γ-32P ATP for one hour at 37° in the presence of Mg++. Incorporation was 90% complete after one hour and approached 0.85 equivalents of label per mole of spectrin (assuming spectrin as 20% of the membrane protein with a molecular weight of 220,000). The rate of incorporation was equal for the two spectrin components. The rate-limiting step did not depend directly on the turnover of the intermediate, but was sensitive to pre-incubation with ATP. Incorporation was inhibited 30% by 0.5 mM Ca++, eliminated by 2.5 mM EGTA, and slightly enhanced by 2.5 mM Ca++. Breakdown of the pre-formed intermediate was not stimulated by adding Ca++. Red cells from three patients with Hereditary Spherocytosis (HS) showed a major decrease (50%) in the extent of 32P incorporation. Also, stimulation of incorporation by cAMP, which is observed in normal membrane preparations was reduced 85% in HS.

These observations in spherocytes suggest a possible role for the spectrin component in the maintenance of normally shaped erythrocytes.

62.

ISOLATION AND CHARACTERIZATION OF AN ABNORMAL HUMAN INTRINSIC FACTOR. Katz, M.5 and Allen, B. H. Royal Victoria Hosp., Montreal, and Washington Univ. Sch. of Med., St. Louis, Mo. A patient has been described (NEJM 287:1425) with B12 deficiency due to a functionally abnormal intrinsic factor (IF). We have now isolated IF from the patient and both parents, who are first cousins, using affinity chromatography (JBC 248:3660). Patient IF appeared normal in terms of: 1) B12 binding (as determined by equilibrium dialysis, absorption spectra, inhibition by pernicious anemia-blocking antibody and displacement of B12 and pseudo-B12 by Co57B12; 2) molecular weight; 3) total amino acid binding (as determined by equilibrium dialysis, absorption spectra, inhibition by pernicious anemia-blocking antibody and displacement of B12 and pseudo-B12 by Co57B12; 3) total amino acid and carbohydrate composition; and 4) immunodiffusion and precipitation with rabbit anti-patient and anti-normal IF. After adsorption with normal IF, however, anti-patient IF sera precipitated the various IFs as follows: patient IF (100%), mother and father IF (50%) and normal IF (0%). Additional adsorption with either mother or father IF completely inhibited the precipitation of patient IF. The association constant determined for patient IF-B12 and human ileal homologs (0.1 x 10⁹ M⁻¹) was 60-fold lower than that determined with normal IF-B12 (6.0 x 10⁹ M⁻¹). The value obtained with both mother and father IF-B12 (3.0 x 10⁹ M⁻¹) was equal to that predicted for, and observed with, an equal mixture of patient and normal IF-B12. These in vitro studies are supported by multiple Schilling tests (0.5 μg B12), performed with a totally gastrectomized volunteer, which gave the following mean urinary excretions of Co57B12: free B12, 0.5%; + patient gastric juice (GJ), 1.8% (P <.001 vs. free B12); + mother or father GJ, 17%; + normal GJ, 30%. These studies demonstrate that the patient is homozygous, and the mother and father are heterozygous, for a structurally abnormal IF that has a decreased affinity for ileal IF-B12 receptors. These studies also indicate that the B12 and binding sites are located on different portions of the IF molecule.

ANEMIA

63.

ABNORMAL FOLATE BINDING PROTEIN(S) IN AZOTEMIC PATIENTS. Hines, J. D., Kamen, B., and Caston, D. Case Western Reserve University School of Medicine, Cleveland, Ohio. Waxman and Schreiber have previously reported on the detection of high concentration folic acid binding protein (FABP) in various tissues including serum of patients with folate deficiency. Utilizing the radiochemical purified hog kidney assay of Kamen and Caston, we have documented a macromolecular FABP in 10 patients with chronic renal disease and azotemia, all of whom manifested anemia, megaloblastic marrow maturation, and macrocytic red cell indices. Four patients were investigated of whom 3 had "saturated" FABPs and one detectable "free" FABP. Serum L. casei folates were between 4.9 and 9.9 ng/ml. Radiochemical assay for free serum folate was less than 1.1 ng/ml. One patient was treated with 0.2 mg of PGA for 2 weeks without any hematologic response. All 5 patients were treated with 5 mg of PGA per day times 2 weeks. During this time there was a significant increase in the reticulocyte count, a slight increase in hemoglobin, and a reversion of bone marrow maturation to normoblastic. These results are indicative that FABP protein(s) detected in azotemic subjects may have pathophysiological significance contributing to the anemia and abnormal cellular maturation of the marrow. These results supply an alternative explanation of the results of Jennett and Goldman showing that azotemic serum inhibits folate transport in an in vitro tumor model.
show avid intestinal absorption of iron which diminishes during weaning. The adult pattern of absorption is
groups. The weaning period (15-25 days) is associated with a rise in Hb and a second growth spurt, but after
show a progressive fall in Hb until the 0th day. Mean body iron concentration at birth is 37 μg/g in sb
increased dietary or stored iron.
thesis of a defect in the regulation of iron absorption in sb, with inappropriate reduction in response to
then established and the characteristic mababsorption of iron in
weaning i.2 mice become rapidly anemic and their growth rate is reduced. Suckling mice of both groups
absorption of radio-iron in inorganic form (dose 0.04 μg/g body weight) and milk iron, from radio-iron
serially. Total body irons were determined by wet digestion. The intestinal
stainable iron were determined
the effects of the gene at earlier stages of development, systematic studies have been made in affected
State University of N.Y. at Buffalo, and Buffalo General Hospital, Buffalo, New York
by endocytosis.
neither organelles nor organized structure. These findings suggest that vacuolization induced
ple surface invaginations and contiguous cytoplasmic vacuoles of varying size which contained
Membranes of basophilic and polychromatic erythroblasts showed marked convolution with multi-
light microscopy indicated that vacuoles were formed by surface invagination (endocytosis).
vacuoles were observed within cytoplasm and nuclei of alcohol
incubated erythroid and myeloid precursors, but not in control cells. In some cells macrophino-
cytosis was evident on light microscopy. Electron microscopy of marrow from patients with
acute alcoholism showing typical alcohol vacuolization in erythroid and myeloid suppression
in acute alcoholism has remained obscure. The vacuoles, usually cytoplasmic but occasion-
ally intranuclear, are well demarcated, often multiple, and have been demonstrated to be PAS
and sudan-red negative. Alcohol vacuolization is reversible and disappears 3-13 days following
cessation of alcohol ingestion but may recur on re-exposure to alcohol. To determine the mech-
amism of vacuole formation, bone marrow specimens from non-alcoholic patients were cultured
in F-12 nutrient medium at 37°C with varying concentrations of ethanol up to 500 mg%. Following
24-48 hours of incubation, vacuoles were observed within cytoplasm and nuclei of alcohol
incubated erythroid and myeloid precursors, but not in control cells. In some cells macrophino-
cytosis was evident on light microscopy. Electron microscopy of marrow from patients with
acute alcoholism showing typical alcohol vacuolization in erythroid and myeloid precursors on
light microscopy indicated that vacuoles were formed by surface invagination (endocytosis).
Membranes of basophilic and polychromatic erythroblasts showed marked convolution with multiple
surface invaginations and contiguous cytoplasmic vacuoles of varying size which contained
neither organelles nor organized structure. These findings suggest that vacuolization induced by
alcohol in erythroid and myeloid precursor cells is due to the direct effect of alcohol
upon the cell membrane leading to localized membrane “irritability” and vacuole formation
by endocytosis.

MECHANISM OF ALCOHOL INDUCED VACUOLIZATION IN HUMAN BONE MARROW CELLS. Young, K.Y.* Klug, P.P.*
Vacculization of myeloid and erythroid precursors in a well known entity occurring in patients acutely intoxicated or in early withdrawal from acute alcoholism. Although frequently encoun-
tered, the mechanism inducing such vacuolization and its role in erythroid and myeloid suppression
in acute alcoholism has remained obscure. The vacuoles, usually cytoplasmic but occasion-
ally intranuclear, are well demarcated, often multiple, and have been demonstrated to be PAS
and sudan-red negative. Alcohol vacuolization is reversible and disappears 3-13 days following
cessation of alcohol ingestion but may recur on re-exposure to alcohol. To determine the mech-
amism of vacuole formation, bone marrow specimens from non-alcoholic patients were cultured
in F-12 nutrient medium at 37°C with varying concentrations of ethanol up to 500 mg%. Following
24-48 hours of incubation, vacuoles were observed within cytoplasm and nuclei of alcohol
incubated erythroid and myeloid precursors, but not in control cells. In some cells macrophino-
cytosis was evident on light microscopy. Electron microscopy of marrow from patients with
acute alcoholism showing typical alcohol vacuolization in erythroid and myeloid precursors on
light microscopy indicated that vacuoles were formed by surface invagination (endocytosis).
Membranes of basophilic and polychromatic erythroblasts showed marked convolution with multiple
surface invaginations and contiguous cytoplasmic vacuoles of varying size which contained
neither organelles nor organized structure. These findings suggest that vacuolization induced by
alcohol in erythroid and myeloid precursor cells is due to the direct effect of alcohol
upon the cell membrane leading to localized membrane "irritability" and vacuole formation
by endocytosis.

IRON METABOLISM IN NEWBORN AND SUCKLING MICE; EFFECTS OF THE sla MUTATION. Kingston, P.J.* Bannerman, C.EM.* and Bannerman, R.M. Medical Genetics Unit, Dept. of Med.,
State University of N.Y. at Buffalo, and Buffalo General Hospital, Buffalo, New York
In the adult mouse, x-linked anemia (sla) is due to defective intestinal absorption of iron. To determine
the effects of the gene at earlier stages of development, systematic studies have been made in affected
(sla/Y, sla/sla), carrier and control mice from birth to 60 days of age. Hb levels, body weights and
stable iron were determined serially. Total body iron were determined by wet digestion. The intestinal
absorption of radio-iron in inorganic form (dose 0.04 μg/g body weight) and milk iron, from radio-iron
labeled mothers, was determined by whole body counting. Newborn sla mice have identical mean birth
weights but lower mean Hb levels than control and carrier mice. During rapid postnatal growth all mice
show a progressive fall in Hb until the 10th day. Mean body iron concentration at birth is 37 μg/g in sla
and 45 μg/g in normal mice; falling to approximately 30 μg/g at 5 days and 25 μg/g at 10 days in both
groups. The weaning period (15-25 days) is associated with a rise in Hb and a second growth spurt, but after weaning sla mice become rapidly anemic and their growth rate is reduced. Suckling mice of both groups
show avid intestinal absorption of iron which diminishes during weaning. The adult pattern of absorption is
then established and the characteristic malabsorption of iron in sla mice is seen; normal mice retaining 22%
and sla mice 6% of the test doses. These changes can be delayed by weaning onto an iron deficient milk
diet. The results illustrate the interrelationship between iron metabolism and growth, and support the hypo-
thesis of a defect in the regulation of iron absorption in sla, with inappropriate reduction in response to
increased dietary or stored iron.

Serum ferritin has been proposed as a means of evaluating iron balance. This study is an attempt to define its broader clinical meanings. In 32 patients with uncomplicated iron deficiency anemia, the serum ferritin averaged 3 ng/ml in contrast to the normal mean of 59 (± 1 SD, 26-133) ng/ml. Iron overload resulted in increased levels as high as 15,000 ng/ml. In 20 patients receiving multiple transfusions, a linear relationship existed between the ferritin level and the number of transfusions administered. Elevations in serum ferritin were also observed in both inflammation and liver disease. In 40 patients with active inflammation, mean serum ferritin was 532 ng/ml. Thus the inflammatory state which produces changes on the amount of body iron but also on the functional behavior of these two cell systems.

In all clinical disorders examined, however, the ferritin level was proportional to iron stores as measured by an iron stain of aspirated marrow. Thus the patient with inflammation or hepatic disease had a higher ferritin level than his normal counterpart at the same level of iron stores. Considering the effects of inflammation (which is known to alter reticuloendothelial activity) and of hepatic damage (the hepatocyte is the receptor for plasma ferritin), the plasma ferritin level may reflect the flow of iron between the reticuloendothelial cell and the hepatocyte. The precise level of plasma ferritin appears to be dependent not only on the amount of body iron but also on the functional behavior of these two cell systems.

SELECTIVE UPTAKE OF RADIOIODIN IN VIVO FROM TRANSFERRIN AT PHYSIOLOGIC IRON SATURATION. Awai, M., Chipman, B.*, and Brown, E.B. Washington University School of Medicine, St. Louis, Missouri 63110.

We have presented evidence from in vivo studies in rats that release of iron from the two binding sites of transferrin to receptors in tissues is a selective process. Past studies employed serum transferrin 90% saturated with radioiron, a level outside the physiologic range. The present studies utilized rat serum transferrin selectively labeled with 59Fe and 55Fe at 35% and 50% saturation levels. To serum from iron deficient rats was added 59Fe as ferric ammonium sulfate to 90% transferrin saturation. Repeated incubations with rat reticulocytes reduced the transferrin 59Fe saturation to 25%. 55Fe was added to the transferrin solution to 35 or 50% saturation levels, and this selectively labeled transferrin was injected into normal and pregnant rats which were killed at various time intervals for measurement of the ratios of the two isotopes in selected tissues. Predominance of 55Fe was present in red cells, marrow and spleen heme, and fetal tissues, while 59Fe was observed in liver parenchymal cells and the mucosa of proximal small intestine. Polarization of 55Fe/59Fe uptake from the transferrin at physiologic iron saturation was 10-20 times greater than with transferrin at 90% iron saturation. These results confirm and extend the concept that tissue receptors remove iron from specific sites on the transferrin molecule and that transferrin plays an active role in the distribution of iron within the body. They suggest that iron receptor sites on cell membranes may identify and remove a single iron atom from transferrin more specifically than from fully saturated transferrin.
THE DEVELOPMENT OF NEW IRON CHELATING DRUGS. Graziano, J.H.*, Grady, R.W.* and Cerami, A.

The Rockefeller University, N.Y., N.Y. At the present time deferoxamine (DF) is the only iron chelating drug that is clinically acceptable for the continual removal of iron deposits resulting from transfusion-induced hemosiderosis. One of its drawbacks is that it must be administered i.m. To obviate this and other problems we have initiated a program to design and evaluate new iron chelating drugs that can be administered orally. Hypertransfused rats receiving the equivalent of 6 blood volumes of heated rat blood were the experimental model. The test drugs were administered either i.p. or p.o. to 6 rats and the urine and feces collected. The samples of urine and feces were ashed in a Muffle furnace at 550° C, dissolved in 3N HCl and analyzed for the amount of iron by atomic absorption. From the compounds that have been tested to date 2,3 dihydroxybenzoic acid (DHB) appears to show particular promise. Approximately 70% of an oral dose (25 mg/kg) is excreted in the urine carrying with it approximately 100 µg of Fe. This amount is comparable to that mobilized by 100 mg/kg of DF, i.p. It is not clear what stores this iron is mobilized from since the administration of larger amounts (up to 300 mg/kg) of DHB do not result in a substantial increase in the amount of the Fe mobilized. After the iron is removed by the chelator, the site is eventually refilled with Fe since subsequent DHB administration results in comparable amounts of Fe mobilized. Preliminary pharmacological and toxicological studies of DHB have not revealed any toxic effects. A series of mice have been injected with 300 mg/kg/day for 6 months without any apparent ill effects. DHB does not remove Fe from animals with normal Fe stores or cause the loss of Ca, Mg, Zn, Cu, Na and K. If further toxicological studies do not reveal toxicity, a limited clinical evaluation will be undertaken.

71. VI. HEMOSTASIS AND THROMBOSIS II

INHIBITION OF NORMAL BLOOD CLOTTING BY ANTI-KALLIKREIN ANTIBODY. Saito, H.* and Ratnoff, O.D. University Hospitals of Cleveland and Case Western Reserve University, Cleveland, Ohio. Hathaway, W.E. et al. (1965) described a new familial clotting defect (Fletcher trait) characterized by a prolonged kaolin partial thromboplastin time (PTT) despite normal levels of the then recognized clotting factors. They postulated that the plasma of affected individuals was deficient in a clot-promoting agent designated Fletcher factor. Recently, Wuepper reported that Fletcher trait plasma was functionally and antigenically deficient in a plasma prekallikrein, presumably the proposed Fletcher factor. To examine the role of a plasma prekallikrein (or kallikrein) in normal blood clotting, we tested the effect of a rabbit antibody, prepared by immunizing New Zealand albino rabbits with purified human plasma kallikrein (specific activity: 755 µM MeOH released/mg protein/hr in a TAMe esterase assay) and rendered monospecific by absorption with Fletcher trait plasma. Incubation of normal human pooled plasma with anti-kallikrein at 37°C for 60 min prolonged the kaolin PTT from a control value of 67 sec to 298 sec, whereas the thrombin time, prothrombin time and RVV time were unaffected. The prolonged kaolin PTT was not due to the neutralization of Hageman factor (F XII), PTA (F XI), Christmas factor (F IX) or AHF (F VIII), since no changes were found in the levels of these clotting factors. Fletcher factor activity, assayed on a substrate of Fletcher trait plasma, was completely abolished, indicating that anti-kallikrein impaired blood clotting by depleting Fletcher factor activity. These data support the view that plasma kallikrein is required in vitro for the intrinsic pathway of coagulation.


The study of a large kindred from Eastern Kentucky with unusually high incidence of venous thrombosis and pulmonary embolism revealed a marked deficiency of antithrombin III (AII) in blood of 9 members in 3 consecutive generations. Biochemical defect, inherited as an autosomal dominant, was assessed by a reduced ability to neutralize both thrombin and factor Xa in plasma fractions obtained by gel filtration on Sephadex G-150 and located in the AII region. Titres of AII in the affected members ranged from 23 to 49% of normal values and were associated with a decreased responsiveness to heparin in vitro. When blood clottability after heparin injection was followed by conventional laboratory tests, discrepancies recorded between deficient subjects and normal volunteers were not uniform. However, the evaluation of post-heparin AII activity revealed an outstanding difference between normal and deficient plasma, reflecting biological stimulation of AII by heparin. Therapy with warfarin in 5 affected members significantly increased the level of circulating AII and contributed to a remarkable increase of residual AII in serum. These findings substantiate the biological role of AII in support of blood fluidity and suggest that stoichiometric binding of this antiproteinase to activated coagulation enzymes prevails in vitro and in vivo.
INHERITED ANTITHROMBIN III DEFICIENCY ASSOCIATED WITH MULTIPLE THROMBOEMBOLIC PHENOMENA.

Shapiro, S.S., Prager, D. and Martinez, J. Cardeza Foundation, Department of Medicine, Jefferson Medical College, Philadelphia, Pa., and Allentown General Hospital, Allentown, Pa.

A family has been studied six of whose members, in three generations, have been found to have plasma antithrombin III levels of approximately 50% of normal (n1 ± 2SD: 100 ± 24%), measured either by a heparin cofactor assay or by single radial immunodiffusion (in the presence and absence of heparin). Three of these individuals, males over 30 years old, have histories of multiple, documented episodes of thrombopelitis and pulmonary embolism. The other three individuals, two males and a female 12 years old or younger, have had no histories of thromboembolism. Affected individuals show only a single antithrombin III band of normal mobility by immunoelectrophoresis and electrophromunofixation, using two different antisera. Plasma levels of α2-macroglobulin and γ1-antitrypsin are normal. Platelet adhesiveness was normal and serum fibrinogen-related antigen was 3 μg/ml in the three symptomatic individuals. Routine coagulation studies were normal except in the one individual who has been on long-term coumarin therapy. Radioactive prothrombin and fibrinogen turnover studies were performed on three affected family members: two symptomatic and one asymptomatic. Fractional catabolic rates for prothrombin were 25, 41, and 43% (n1 ± 2SD: 42.5 ± 12.4% of plasma pool/day). Fractional catabolic rates for fibrinogen were 18, 27, and 24% (n1 ± 2SD: 23.9 ± 7.5% of plasma pool/day), the first value in each series being the anticoagulated family member. Intravascular-extravascular distribution of both proteins was in the normal range. It appears that, although antithrombin III deficiency in this family is associated with severe thromboses there is no detectable increase in prothrombin or fibrinogen metabolism.

OPTICAL ENANTIOMORS OF SODIUM WARFARIN IN WARFARIN-SENSITIVE AND WARFARIN-RESISTANT MAN.

O'Reilly, R.A., Santa Clara Valley Medical Center, San Jose, and UCSF School of Medicine, San Francisco, California. All sodium warfarin used clinically is a racemic mixture composed equally of two optical enantiomers. Ten normal subjects were administered the resolved enantiomers in separate experiments as single oral doses of 1.5, 0.75, and 0.375 mg/kg body weight and daily blood levels of the one-stage prothrombin time (Quick) and of the warfarin levels spectrophotometrically were performed. The plasma T1/2 of S(-)-warfarin was 3.3 ± 0.4 hours (mean ± SEM) and of R(+)-warfarin was 58 ± 4 hours, yet the intrinsic hypoprothrombinemic activity of the S(-)-warfarin was 3.4 times greater. On long-term therapy the average daily dose in the normal subjects for R(+), S(-), and racemic (±)-warfarin was 25, 6, and 7 mg, respectively, while in the propositus of the second reported kindred with warfarin resistance, the average daily dose was 250, 60, and 75 mg, respectively. The same 4 to 1 hypoprothrombinemic activity ratio for R(+)- to S(-)-warfarin was found in both warfarin-sensitive and warfarin-resistant man, which is consistent with the data found utilizing racemic warfarin in previous studies. Thus, the mechanism for warfarin resistance in man is not a pharmaco-kinetic one but must be located at the receptor site. It is concluded that despite its short half life in plasma the hypoprothrombinemic activity of (±)-warfarin, probably because of its greater intrinsic activity at the receptor site for oral anticoagulants.

ACTIVATION OF BOVINE PROTHROMBIN-FEED BACK REGULATION. Bajaj, S.P.*, Butkowski, R.J.*, Fass, D.N. and Mann, K.G. Mayo Clinic, Rochester, Minn.

The activation of bovine prothrombin (II) to α-thrombin (α-IIa) proceeds by the successive removal of amino terminal segments giving rise to a number of single chain polypeptide intermediates (Int.). The first step catalyzed by either factor Xa or IIa yields the amino terminal segment Int. 1. Int. 1 is then cleaved by factor Xa into an amino terminal segment Int. 2 and the carboxyl terminal Int. 1. Int. 1 and Int. 2 are precursors of α-IIa while Int. 3 and Int. 4 are "pro" fragments. Equilibrium dialysis studies indicate that prothrombin has 9 Ca2+ binding sites which can be differentiated in terms of binding affinity. All the five strong Ca2+ binding sites are contained within the Int. 3 segment of the molecule. Int. 1 and Int. 2 which lack the Ca2+ binding Int. 3 segment (of II) are rendered poor substrates for XaCa2+ phospholipid complex activation when compared to II. Int. 1 and Int. 2 also activate much more slowly than II in the 2-stage assay system for II. Int. 3 is an inhibitor in the one-stage prothrombin time assay as well as in purified II activation systems containing factor Xa, Ca2+ and phospholipid. Int. 4 on the other hand, markedly shortens the partial thromboplastin time (PTT) but has virtually no affect on the one-stage prothrombin time. Int. 4 also increases the esterase activity of IIa towards tosyl-L-arginine methyl ester whereas the clotting activity of IIa is unaffected. These studies suggest that the intermediates of II activation serve as feed back regulators-Int. 3 acting as an inhibitor of II activation and Int. 4 as an activator at some step in the intrinsic coagulation pathway which precedes prothrombin activation.
ACTIVATED PROTHROMBIN CONCENTRATE FOR PATIENTS WITH FACTOR VIII INHIBITORS. Kurczynski, E.M.* and Penner, L.A. Dept. of Pediatrics and Simpson Memorial Institute (Dept. of Internal Medicine) University of Michigan Medical Center, Ann Arbor, Michigan. Management of the hemorrhagic manifestations of the Factor VIII inhibitors encountered in hemophilia and as an acquired disorder has been difficult and in many cases impossible. We have recently used activated prothrombin concentrate (Auto-IX, Hyland), a purified prothrombin complex, which contains high levels of "activated" Factor IX and X, in addition to prothrombin, VII and other prothrombin intermediate products. Five patients who did not respond to massive doses of purified Factor VIII concentrates were treated with the product for serious or life-threatening bleeding episodes. Rapid cessation or marked diminution of bleeding was achieved. Four were boys with classic hemophilia A with inhibitor levels ranging from 6 to greater than 50 units. The fifth was a 58 year-old woman with an acquired inhibitor of 100 units. There were no significant side effects, and no clinical evidence of hypercoagulability following 12 infusions of the activated prothrombin. Improvement in PTT values usually was achieved immediately and continued to be observed up to 24 hours afterwards. In the patient with the acquired inhibitor, rebleeding occurred 24-48 hours later, and a repeat administration was necessary. Cessation of bleeding was noted following each dose. Activated prothrombin complex appears to be an effective and safe means of treatment for patients with Factor VIII inhibitors who develop life-threatening episodes of bleeding.

EFFECT OF LIPID ON NEUTRALIZATION OF ACTIVATED FACTOR X BY ITS PLASMA INHIBITOR. Yin, E.T.*, Chen, J.S.* and Wessler, S. Jewish Hospital and Washington University Medical Center, St. Louis, Missouri. We have previously demonstrated, in rabbits, that phospholipids augment the intensity and duration of the thrombogenicity of infused activated Factor X (Xa); and, in vitro, that plasma activated Factor X inhibitor (XaI) inactivates Xa, a reaction accelerated by trace quantities of heparin. The present investigation attempts to elucidate the in vitro effect of lipid on the XaI-Xa reaction in the presence and absence of heparin. When 4 units of Xa are incubated with 7.5 μg of XaI; 60% of Xa activity is neutralized in 30 minutes. If, to this reaction mixture, are added 50 μg of an equimolar mixture of phosphatidyl serine and phosphatidyl choline, and 4mM CaCl2; 100% of Xa activity persists for 30 minutes. More lipid is required to maintain the stability of Xa when inhibitor concentrations exceed 20 μg. When, however, 0.1 unit of heparin is added; 98% of the Xa activity is inactivated in 3 minutes. The heparin requirement is dependent on the concentration of lipid in the reaction mixture. These data define new roles in blood coagulation for lipid - its interference with the XaI-Xa reaction, per se, as well as with the response of this reaction to heparin.

CIRCULATING ANTICOAGULANT INTERFERING WITH THE ACTION OF FACTOR XIa IN LUPUS. Krieger, H.* and Breckenridge, R.T. University of Rochester and Rochester General Hospital, Rochester, New York. Proteins which interfere with blood coagulation have often been described in patients with disseminated lupus. The most frequent type interferes with the conversion of prothrombin to thrombin and thus prolongs the prothrombin time. Infrequently patients exhibit anticoagulants which appear to block the earlier stages of coagulation such as those involving Factor VIII or the formation of activated factor X. We have recently studied a patient with a "lupus-like syndrome" who demonstrated a prolonged partial thromboplastin time and a normal prothrombin time. By means of a sequential clotting system, utilizing crude coagulation factors, it was possible to demonstrate that this patient's anticoagulant inhibited the action of factor XIa during the conversion of Christmas factor to its active form. Similar to the other anticoagulants in lupus, this particular anticoagulant was shown to be a gamma globulin of the IgG class. It is concluded from this study that patients with disseminated lupus manifest a variety of hemostatic abnormalities including an occasional patient who demonstrates inhibition of coagulation during its earliest stages.
THE SUBUNIT STRUCTURE OF FIBRINOGEN (Fbg) BALTIMORE AND ITS FIBRIN (f). Brown. C.H., III and Crowe, M.F.* Baylor College of Medicine, Houston, Texas. Fbg Baltimore differs from normal Fbg in its anodal electrophoretic migration, increased rate of clotting (partially corrected by acid pH or calcium) and delayed release of fibrinopeptide A (Beck, et al. J.C.I. 50:1874, 1971). We have examined the subunit structure of Fbg Baltimore and of thrombin-induced clots containing f-Baltimore formed at various times from 5 min to 24 hr using SDS-polyacrylamide gel electrophoresis. With this method, which separates proteins by their molecular weight, McKee, et al. (Proc. Nat. Acad. Sci. 66:738, 1970) showed that normal Fbg and non-cross-linked f contain α-, β-, and γ-monomers, whereas cross-linked f contains α-polymers, β-polymers and γ-dimers. In the present studies, purified Fbg Baltimore and both non-cross-linked as well as cross-linked f formed at pH 6.4 and 7.4 from plasma obtained from a patient with Fbg Baltimore exhibited normal subunit structure. Utilizing purified Fbg as substrate, non-cross-linked normal f contained α-subunits that evolved during the 24 hr clotting period into smaller units designated α' and α''. The evolution of α' and α'' in non-cross-linked f-Baltimore formed from purified Fbg Baltimore was abnormal and varied according to pH conditions. Cross-linked f-Baltimore formed from purified Fbg Baltimore at pH 7.4 contained α-dimers and α-subunits not present in normal f. More complete α-polymerization in f-Baltimore occurred at pH 6.4. β-Dimerization appeared normal in f-Baltimore. Our conclusions are: (1) The subunit molecular weight of Fbg Baltimore is normal; (2) Plasma that contains Fbg Baltimore and normal Fbg, clotted under the conditions of this study, yields clots containing only normal γ; and (3) α-polymerization (presumably end to end) is defective in f-Baltimore formation. These results further suggest that the defect in Fbg Baltimore resides in the α-chain.

EVIDENCE THAT PLASMIN FRAGMENT D FROM HUMAN FIBRINOGEN HAS A COVALENT DIMERIC STRUCTURE. Mosesson, M.W.* Finlayson, J.S.* and Galanakis, D.K.* S.J.N.Y.-Downstate Med. Ctr., Brooklyn, N.Y. and Bureau of Biol., F.D.A., Bethesda, Md. Human fibrinogen (f) consists of 3 pairs of chains (Aα, Bβ, γ) covalently linked by disulfide bridges to form a dimeric structure. Upon advanced plasmin hydrolysis of f, 2 major types of core fragments, termed D and E, are formed. E is derived from the NH2-terminal region of f and contains portions of all 6 chains covalently linked in a dimeric structure. Recent data indicate that a covalent dimeric structure also exists in the D moiety (whose structure overlaps that of the COOH-terminal region of f). A partially clottable plasmin derivative subfraction (termed I-9D) isolated during various hydrolytic phases was characterized by the presence of substantial amounts of intact Bβ and γ chains (MW 60 400 and 49 400, respectively) but no intact Aα chain (MW 70 900). Its Aα population consisted mainly of Aα remnants (Aα/Aα) of MW 25 000 or less from which COOH-terminal portions had been cleaved. When I-9D was subjected to further plasmin hydrolysis, a single-stranded chain (Aα) was released. Its size (MW 32 000) showed that it must be of Bβ or γ origin; Bβ origin was established by immunological and related analyses. The structure of f precludes the release of a derivative of MW 32 000 from the NH2-terminal region of the Bβ chain, indicating that /β arose from the COOH-terminal portion of the parent chain. Release of fragment E, plus other hydrolytic attack on the core, resulted in advanced forms of fragment D (Dα) which contain a plasmin-resistant γ chain derivative (γ'') (MW 42 000) and smaller remnants of Aα and Bβ origin (MW < 6 700). Since the MW of Dα is not less than 80 000, it must contain both γ chains, thereby implying the existence of a covalent dimeric structure.

AUTOIMMUNITY IN MAN TO THE FIBRIN FRACTIONS OF FIBRINOGEN. Plow, E.F.* and Edgington, T.S. Scripps Clin. & Res. Fdn., La Jolla, CA. The cleavage of fibrinogen and fibrin by plasmin induces significant structural and conformational modifications in these molecules which in turn lead to the generation of unique antigenic expressions specifically associated with the cleavage products. The presentation of such cleavage-associated neoantigenic expressions to a host endowed with immunocompetent lymphocytes could potentially recruit an autoimmune response manifest by the production of autoantibodies with specificity for the cleavage fragments. To determine if such antibodies are present in the normal human population, 125I labeled fibrinogen D fragment (fg-D) was incubated with human sera containing no detectable cleavage fragments. Following an overnight incubation, serum immunoglobulins were precipitated with specific goat antiserum; and binding of 125I fg-D determined. In 46 of 50 sera (92%), low levels of fg-D binding could be detected. This binding could be specifically inhibited by the addition of unlabeled fg-D but not by fibrinogen; and isolated 75 fractions from several sera retained their capacity to bind 125I fg-D. Binding was independent of the total concentration of immunoglobulins in the individual sera or of the concentrations of IgG, IgA, or IgM; and nonspecific binding of fg-D to immune complexes could not be demonstrated. Utilizing other purified radiolabeled fragments, qualitatively similar binding of the X and Y fragments but not of the E fragment could be demonstrated. In 10 plasmas containing cleavage fragments no binding of fragments could be detected. The ability of immunoglobulins to bind cleavage fragments appears to be a new interface between the coagulation and immune systems, which may be significant in controlling the numerous pathobiologic activities of the cleavage fragments. In addition, complexes of autoantibody and cleavage fragments may be of pathogenetic significance in disease.
82. COAGULATION, FIBRINOGEN SURVIVAL, AND FIBRIN SPLIT PRODUCTS IN SICKLE CELL DISEASE. 
Maluf, R., Steiner, T. and Siegel, M.S., Boston Sickie Cell Center, Boston Medical Hospital, and Tufts Medical School, Boston, Massachusetts. The possible participation of blood coagulation in sickle cell disease has been investigated in 16 patients with SS hemoglobin, 4 patients with SC hemoglobin, and 1 patient with S-Thal. There were 8 patients in crisis, 20 out of crisis (6 studied both in and out of crisis). 

Factors I, II, V, VII, VIII, IX, X, XI, thrombin time, partial thromboplastin time, fibrin split products, fibrinogen turnover, plasminogen, platelet count and platelet aggregation were done. Furthermore, the injection of 125I labelled fibrinogen was followed by a sequential surface counting in each subject for the purpose of determining local depositions of fibrin. Detectable amounts of fibrin split products were found only in 4 (2 normal, 2 elevated levels) out of 22 samples from patients out of crisis, and in 2 (both elevated) out of 9 samples from patients in crisis. Fibrinogen turnover compared to normal volunteers (T1/2=4.3 days) was found considerably shortened (T1/2=2.7 days) in 5 patients in crisis (P<0.05) as well as in 7 patients out-of-crisis (T1/2=3.1 days). Surface counting showed increased radioactivity in lungs of patients when compared to normals. Prolonged prothrombin time, decreased factor II, VII and X were almost constant findings in the patients. Liver impairment may explain low levels of K-dependent coagulation factors. Shortened fibrinogen survival, apparent fibrin accumulation in the lungs, and tendency for low plasminogen and K-dependent coagulation factors, however, may point to intravascular coagulation. If so, there is a unique disparity between these findings and a notable absence of fibrin split products in most patients.

83. VII. IMMUNOHEMATOLOGY, TRANSFUSION, AND TRANSPLANTATION

IMMUNOLOGIC FUNCTION AND TUMOR SPECIFIC ANTIGENS (TSA) IN CANINE MALIGNANCIES. Weiden, P.L.*, Tsol, M.S.*, Kolb, H.J.*, Ochs, H.D.*, Kold, H.*, Lerner, K.L., Graham, T.C.*, Thomas, E.D., and Storb, R., University of Washington, School of Medicine, Seattle, Washington. We have previously demonstrated the dogs with lymphosarcoma (LSD) have uniform histological and clinical features and are a suitable model for spontaneous malignancy in a randomly bred species (Blood, 37:340, 1971). The immune status of 62 untreated LSD and the clinical features and immune status of 35 dogs with spontaneously occurring solid tumors (STD) have been investigated. 

Studies of humoral immunity revealed low IgG levels in LSD but not in STD. Hemagglutinin titers following sheep RBC immunization were suppressed in LSD and normal in STD. Similar findings were obtained following primary and secondary immunization with bacteriophage. Cellular immunity, assessed by first and second set allogeneic skin graft survival, was impaired in LSD but not in STD. Response to PPD challenge following sensitization with BCG and in vitro lymphocyte blastogenesis (LB) following stimulation by phytohemagglutinin or allogeneic lymphocytes were deficient in both LSD and STD.

TSA were sought using autochthonous tumor cells to stimulate LB. Peripheral lymphocytes were cultured with irradiated, autochthonous tumor cells, either freshly prepared or cultured overnight, and with unirradiated, autochthonous lymphocytes (control). A tumor/control ratio of >2.0 was considered positive. 16/25 LSD and 12/16 STD tested were positive with tumor/control ratios up to 26. These results demonstrate that LSD have a marked deficiency of immunologic function, while STD are relatively intact immunologically. TSA can be demonstrated in both groups by LB stimulated by autochthonous tumor. These findings in untreated dogs are of importance in designing immunotherapy protocols.


We have previously reported the detection of LAA on human leukemic cells using primate anti-human leukemia sera (Science 178:986, 1972). These antisera demonstrated an antigen associated with CLL and ALL cells and another antigen(s) associated with CGL or AML cells. The results are contrary to data reported by others obtained with rabbit anti-human leukemia sera. Subcellular leukemic antigens from CLL, CGL and AML cells were prepared by trypsin digestion and further purified by Sephadex chromatography. This material was used to produce rabbit antisera which reacted strongly (>1:80 cytotoxicity) with normal human blood and all leukemic cells tested. After absorption with normal cells, rabbit anti-CLL serum reacted only with CLL, ALL, CGL, AML and circulating white blood cells of non-Hodgkin's lymphoma. Absorption of the rabbit antisera with leukemic cells from some AML, CGL or CLL donors removed reactivity for cells from all types of leukemic donors tested. LAA activity can be removed from the cell surface by treatment with trypsin or neuraminidase. Inhibitors of protein synthesis, but not inhibitors of nucleic acid synthesis, prevent reappearance of LAA(s).

Mitogen-stimulated lymphocytes, WBC's of family members of leukemias, human thymocytes, fetal tissues, cord lymphocytes and tissue culture cell lines derived from non-leukemic donors do not have detectable LAA. However, a primate myelogenous leukemia cell line and peripheral cells from a monkey with radiation-induced myelogenous leukemia have LAA. The combined use of primate and rabbit anti-human leukemia sera reveal that there is a wide spectrum of LAA. Some LAA are specific for the morphological types of leukemia while other LAA(s) detected by rabbit antisera are cross-reactive. Some primate leukemia cell lines have a human LAA.
85.

USE OF CHEMICALLY MODIFIED LYMPHOMA CELLS TO PRODUCE IMMUNITY IN AN UNRESPONSIVE TUMOR-HOST SYSTEM. Prager, M.D.*, Baechtel, S.**, Ribble, R.J., and Ludden, C.M.** The University of Texas Southwestern Medical School, Dallas, Texas. Active immunization with chemically modified cancer cells can protect syngeneic hosts against challenge with the same tumor. In certain cases chemical modification has been superior to x-irradiation. Conceptually modified cells might be applied in tumor-host systems characterized by either of 2 types of immunologic relationship: (1) response by host against progressively growing tumor, or (2) host unresponsiveness. The C3H mouse-6C3HED lymphoma represents a type I system for which the efficacy of modified cells in producing immunoprophylaxis has been amply demonstrated. Using a standardized regimen of 3 weekly vaccinations with 10^9 modified cells 1 week later by a 10^3 tumor cell challenge, there was no protection in the BALB/c mouse-P1798 lymphoma type 2 system. P1798 cells were altered by x-rays, sulfhydryl blocking agents, neuraminidase, other glycosidases, the mitogens PHA and Con A, NaIO_4, fluorodinitrobenzene, polypropyleneimine, and combinations of these agents. An allogenic antiserum raised in C57Bl/NSJ mice (H-2^b), after absorption, gave immunofluorescent reactions with P1798 but not normal BALB/c (H-2^d) lymphoid elements. With a tumor specific antigen having been demonstrated, long term immunization with iodoacetamide modified P1798 cells was undertaken. After 12 vaccinations over 4-5 months, 45-94% of BALB/c mice were tumor free at least 60 days after challenge. The importance of this result is in establishing the principle that even in an unresponsive tumor-host system it is possible to effect immunity with chemically modified cells.

86.

EFFECT OF PRIOR BLOOD TRANSFUSIONS ON MARROW GRAFTS: ABROGATION OF SENSITIZATION BY PRO-CARBAMINE (PRO) AND ANTIHYMOCYTE SERUM (ATS). Storb, R., Weiden, P.L.*, Floersheim, G.L.*, Noll, H.J.*, Graham, T.C.* and Thomas, E.D., University of Washington, School of Medicine, Seattle, Washington. A single blood transfusion can immunize a canine recipient and lead to rejection of a subsequent marrow graft even when donor and recipient are "histocompatible" littermates. We evaluated whether immunization by prior transfusion could be abrogated by PRO and/or ATS. Recipients were given 1200 R irradiation followed by marrow from histoincompatible unrelated donors. GROUP 1: 7 dogs not given prior transfusions had sustained marrow engraftment as previously defined (J. Immunol., 105:627,1970). Dogs in GROUPS 2-6 were transfused with blood from the marrow donor on days -20 and -13 before irradiation. GROUP 2: 7/11 dogs given no further therapy rejected the marrow graft. GROUP 3: 1/10 dogs given PRO, 12.5 mg/kg i.v. on days -8,-6,-4 and ATS, 0.6 ml/kg on days -7,-5,-3, rejected the graft. Nine had sustained engraftment. GROUP 4: 5/10 dogs given ATS only rejected. GROUP 5: 4/8 dogs given PRO only rejected. GROUP 6: 5/5 dogs given PRO and ATS and further donor transfusions on days -6,-4,-2 rejected. In conclusion, immunization by preceding blood transfusion can be abrogated by a combination of PRO and ATS (p<0.025). PRO or ATS alone are not sufficient (p>0.1).

Continuation of donor blood transfusions during the PRO-ATS regimen precludes successful grafts. Only 1 of 5 marrow transplant patients with prior transfusions from family members had a functioning and sustained marrow graft. Two additional patients with similar transfusion histories were given the PRO-ATS regimen in addition to conditioning with cyclophosphamide and/or irradiation. Results were encouraging in that the regimen was well tolerated, and both patients had sustained marrow engraftment.

87.

DETECTION OF HUMORAL PRESSENTIZATION IN PROSPECTIVE RECIPIENTS OF KIDNEY TRANSPLANTS BY CULTURED HUMAN LYMPHOID CELLS. S. Ferrone, A. Ting*, M.A. Pellegrino*, P.I. Terasaki* and R.A. Reifeld*. Scripps Clinic and Research Foundation, La Jolla, California and Department of Surgery, University of California, Los Angeles, California. There is a compelling body of evidence that the presence of lymphocytotoxins in the sera of prospective recipients of kidney transplants unfavorably affects the survival of the subsequent graft. Some of these antibodies can only be detected by increasing the sensitivity of the lymphocytotoxic test either by adding sublytic amounts of rabbit antihuman lymphocyte serum to the reaction mixture, by prolonging the incubation time or by treating target cells with trypsin. Cultured human lymphoid cells are more sensitive target cells than peripheral lymphocytes, because they contain a higher density of cell surface antigens. Twelve cultured human lymphoid cells with different HL-A phenotypes were utilized as targets in the cytotoxic test to screen the sera of graft recipients who had rejected their transplants, although lymphocytotoxic antibodies had not been found in the regular cytotoxic test. As controls, sera from recipients who did not reject transplants and from recipients who rejected their transplants by non-immunologic mechanisms were used. Some of the patients who rejected their transplants by immunologic mechanisms had cytotoxic antibodies against cultured cells. These antibodies activated the classical pathway of the complement system and were directed against determinants different from HL-A, since they were not absorbed by platelets from 70 people. No antibodies were detected in sera from patients who rejected the transplants by non-immunologic means. Thus, cultured human lymphoid cells appear to be useful in detecting humoral presensitization in prospective recipients of kidney transplants.
HIGH RESOLUTION SEPARATION OF BLOOD CELL TYPES BY DENSITY GRADIENT CENTRIFUGATION. Corash, L.* Filip, D.J.* Eckstein, J.D.* and Rivers, S.L. Center for Disease Control and American Red Cross, Atlanta, Georgia. The effect of storage temperature (4°C and 22°C) on platelet function was studied. All studies were performed on platelet concentrate samples prepared in CFP anticoagulant and adjusted to platelet counts of 500,000/μL immediately prior to study.

The concentrations of platelet adenine nucleotides were determined by the firefly luciferase method of Holmsen. After 72 hours of storage at 22°C, there was an 85-90% decrease in the total levels of platelet ADP and ATP, whereas only a 40% decrease occurred in platelets stored at 4°C. The nucleotide loss was progressive at both temperatures during the 72-hour storage period, but the rate of decrease was much greater at 22°C. Aggregation studies showed a good response to exogenous ADP, epinephrine and collagen in the platelets stored up to 72 hours at 4°C. Aggregation was markedly diminished by 24 hours in the platelets stored at 22°C.

Collagen-induced release of platelet ADP was quantitated and paralleled the aggregation results. There was no diminution of platelet factor - 3 activity over 72 hours in platelets stored at 4°C. There was a marked diminution of platelet factor - 3 activity over 72 hours in platelets stored at 22°C. Considerable controversy exists regarding the optimum conditions for storage of platelet concentrates intended for transfusion. These in vitro studies show that storage of platelets at 4°C is associated with superior preservation of platelet adenine nucleotides, platelet aggregation and the release reaction for up to 72 hours.

A QUANTITATIVE ASSAY FOR ANTI-GRANULOCYTE (PMN) ANTIBODIES: PHAGOCYTOSIS OF SENSITIZED PMN BY MACROPHAGES. Boxer, L.A. and Stossel, T.P.* Children's Hospital Medical Center, Boston, Mass. Rabbit alveolar macrophages (RAM) because of their large size, were found capable of ingesting intact human PMN sensitized with anti-PMN antisera. A quantitative assay was devised in which normal human PMN purified by gradient centrifugation were incubated in test sera, washed and added to RAM in balanced salt solution containing nitroblue tetrazolium (NBT). The initial rate of NBT reduction by RAM ingesting the PMN was measured spectrophotometrically. The rate of NBT reduction by RAM was proportional to serum concentration used to sensitize PMN and was negligible with RAM or PMN alone or RAM + unsensitized PMN. The following % changes in NBT reduction are relative to incubations of RAM and PMN treated with autologous (donor) sera:

<table>
<thead>
<tr>
<th>Sources of serum</th>
<th>% change in rate of NBT reduction</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>16 normals, nonimmune neutropenia, ITP, anti-Rh</td>
<td>14 ± 33</td>
<td>NS</td>
</tr>
<tr>
<td>20 frequently transfused patients</td>
<td>193 ± 133</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>5 neonatal isoimmune neutropenia</td>
<td>733 ± 378</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>4 rabbit anti-human PMN</td>
<td>387 ± 170</td>
<td>&lt;.001</td>
</tr>
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The phagocytic activity of the sera was in the IgG fraction. Some sera with opsonic activity did not agglutinate PMN (44%), did not exhibit cytotoxicity in the presence of fresh serum as determined by 51Cr release from labelled PMN (43%), and did not impair the ability of the PMN to ingest particles (30%). The reverse was not found. Thus opsonization was more sensitive than agglutination, cytotoxicity or functional impairment as an indication of antibody activity. The detection of anti-PMN antibody by its opsonic activity is quantitative and represents a model for immune clearance.
91.
QUANTITY AND RH SPECIFICITY OF RBC BINDING SITES FOR α-METHYLDOPA IgG AUTOANTIBODIES.
Masouedis, S.P. U. Calif. San Diego, La Jolla, California. The ultrastructural, two-
dimensional distribution of RBC membrane binding sites of IgG anti-D and of α-methyldopa
(α-MD) IgG autoantibodies was studied to characterize the Rh specificity of α-MD IgG
antibodies. The quantity of IgG anti-D bound to RBC was measured using 1-125 anti-D and the
quantity and spatial disposition of both α-MD IgG and anti-D IgG on the RBC membrane was
ascertained using electron microscopy and ferritin labeled rabbit anti-human IgG with methods
described previously. α-MD IgG binds to D-positive and to a lesser extent to D-negative RBC,
which contain about 30% less sites than the D-positive RBC. The number of α-MD IgG sites on
D-positive RBC was similar to the number of RBC D antigen sites observed with some D-positive
phenotypes of reduced D antigen content (2 to 6000 sites per RBC). The distribution of α-MD
IgG binding sites was random and resembled the pattern of distribution of D antigen sites on
D-positive RBC. There was a reduction in binding of 1-125 anti-D IgG (about 15% with the
conditions used) when D-positive RBC were incubated with both α-MD IgG and 1-125 anti-D IgG.
The total number of IgG molecules bound (anti-D plus α-MD) as determined by electron micros-
copy was 10 to 20% less than would be expected if α-MD IgG and anti-D IgG occupied different
receptor sites. These observations suggest that α-MD IgG binds to sites other than D but
that these sites are Rh-related, since α-MD IgG, under appropriate conditions, can displace
anti-D IgG from D receptor sites. A more precise assessment of the Rh specificity of α-MD
through the use of these techniques may provide an explanation for the inconsistent hemolysis
found in patients on α-MD who develop a positive Coomb’s test.

92.
THE FIXATION OF THE FIFTH COMPONENT OF COMPLEMENT (C5) TO NORMAL AND PAROXYSMAL
NOCTURNAL HEMOGLOBINURIA (PNH) RED CELLS. Rossie, W.P., Adams, J.P* and
Zellinger, M.* Duke University Medical Center, Durham, North Carolina
The fixation of C5 is a critical step in complement (C') mediated lysis
since it establishes the lytic site on the membrane. We have investigated the
fixation of C5 to normal and both types of PNH red cells (the moderately C'-
sensitive population II and the markedly C'-sensitive population III) using a
quantitative anti-C5 absorption test. The amount of C5 fixed when C' is acti-
ved by anti-I is 3-5 times greater on both types of PNH cells than on normal
cells but this is because the fixation of C3, the preceding component, is in-
creased by the same proportion; i.e., the ratio C5/C3 is the same on all cells.
The amount of lysis obtained for a given amount of fixed C5 is 3-5 times greater
than normal for population III but only 1.5-2.5 times greater for population
II, accounting for the difference in susceptibility to lysis by C'. This dif-
ference in effectiveness of membrane-bound C5 was not due to a greater stabil-
ity of the molecule in the membrane of PNH cells. When C' was activated by
acidification of the serum, the same ratio of C5/C3 was found as for lysis by
antibody but when C' was activated by cobra venom factor, the ratio was in-
creased and the C5 which was fixed was hemolytically more effective. These
studies show that the increased lysis of PNH cells is due to the establishment
of more potential hemolytic sites and to an increased fraction of these going
to completion.

93.
NEW ASPECTS OF GUINEA PIG SERUM (GPS) INDUCED HEMOLYSIS OF NEURAMINIDASE TREATED SHEEP RED
CELLS (N-SRBC). P.K.Lauf, Dept. Physiology, Duke University Medical Center, Durham,N.C. 27707
Red cells treated with neuraminidase are hemolyzed by mammalian sera in the absence of any ad-
ded antibody. The factors causing this hemolysis and its mechanism are largely unknown. This
paper reports on recent studies aimed at a) defining the nature of the hemolytic process and b)
characterizing some of the components involved in hemolysis of N-SRBC by GPS. It was found that
N-SRBC became maximally susceptible to hemolysis by GPS when about 80% of the total enzyme hy-
drolyzable neuraminic acid was cleaved. At 37°C and high GPS concentrations hemolysis proceeds
rapidly and is complete within 3-5 minutes after adding GPS to N-SRBC. The effect is strongly
dose dependent with a temperature optimum between 33 and 41°C. Hemolysis is markedly slower at
23°C and absent at 0°C. Further kinetic studies on hemoglobin release and 42K-eflux in N-SRBC
exposed to GPS show that, after a brief lag phase, cation leakage precedes hemoglobin loss by
less than 100 seconds suggesting an osmotic analogue of hemolysis. For this dramatic change of
cation permeability followed by hemolysis presence of GPS is necessary. N-SRBC incubated with
heated sera have normal cation pump and leak fluxes. Absorption studies permit the distinction of at least two classes of factors responsible for hemolysis. One factor can be adsorbed to N-
SRBC at 0°C without affecting the CH50 titer as tested on Forssman antibody sensitized control
SRBC. Such absorbed GPS will not affect N-SRBC, however, it does hemolyze N-SRBC at a much high-
er hemolytic efficiency (order of magnitude) when these cells were previously exposed to high
concentrations of GPS at 0°C followed by washing. The nature of the component which "sensiti-
zes" N-SRBC for the hemolytic action of GPS will be discussed, particularly in light of remov-
ing "non-specific" hemolytic effectors from GPS prior to immune-hemolysis studies.
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94. THE ROLE OF THYMINE-RICH SINGLE-STRANDED ENDS IN THE BINDING OF SERUM ANTIBODY TO DEOXYRIBO-NUCLEIC ACID (DNA). Richard J. Samaha* and William S. Irvin* (intr. by C.E. Mengel). University of Missouri, Columbia, Missouri. Antibodies to native DNA have been found in the sera of patients with systemic lupus erythematosus (SLE). There is conflicting data on the specificity of antibody binding to double-stranded and/or single-stranded DNA. We have evidence that structural defects in purified DNA play a significant role in the binding of nucleic acid to antibody. Tritiated thymidine labeled human cell DNA was fractionated by methylated albumin kieselguhr (MAK) chromatography into a salt elutable fraction (S) composed of primarily double-stranded DNA with small single-stranded regions, and an alkaline elutable fraction (A) composed of double-stranded DNA with significant single-stranded regions. A single-strand specific enzyme removed 70% of the radioactivity, but only 10% of the nucleic acid contained in fraction A, indicating that the single-stranded regions are rich in thymine. By an end specific single-strand enzyme which removed 65% of the radioactivity from fraction A, we determine that the single-stranded regions are located at the ends of the molecules in both fractions. Thus, the S and A fraction DNA differ only in the length of thymine rich single-stranded ends. However, selected SLE sera bound 60% of fraction S but only 30% of fraction A using a Farr technique. These data show that double-strandedness of the DNA is not the major factor in determining the binding of nucleic acid to antibody. Our data suggests that single-stranded ends rich in thymine play a significant role in the binding of SLE antibody to deoxyribonucleic acid.

95. VIII. STEM CELL KINETICS

EFFECTS OF X-IRRADIATION ON THE ENVIRONMENT FOR HEMATOPOIETIC STEM CELL GROWTH. Chamberlin, William and Fried, Walter, University of Illinois Hospitals, Chicago, Illinois. Hematopoietic tissue regenerates in femurs and spleens implanted subcutaneously into isogeneic mice within 6 weeks. The femur implants contain about 500 CFU (colony forming units are measured by their ability to form macroscopic colonies on spleens of irradiated hosts), and spleen implants contain about 50. Thereafter this number remains constant. The implant's CFU have been shown to be of both donor and host type. To determine the effect of depleting the host's CFU population on their growth in implants, BDF1 mice were exposed to 0 or 950 rads (and injected with 10^6 marrow cells). They then were each implanted with a femur and a spleen from a non-irradiated BDF1 donor. Two, 4, and 6 weeks later the number of CFU in the host and in the implanted femurs and spleens were assayed. The number of CFU in host tissue did not return to pre-irradiation level till 6 weeks post-irradiation. Femurs implanted into irradiated hosts contained about 75% more CFU 2 and 6 weeks after implantation than did those in non-irradiated hosts. After 6 weeks there were no consistent differences. Spleens implanted into irradiated hosts contained about 5 X as many CFU as did those in non-irradiated ones 2, 4, & 6 weeks after implantation. In other experiments mice were exposed to 950 rads or 0 rads. Two, 4, & 6 weeks afterwards, femurs were removed and implanted into non-irradiated hosts. Implants from all irradiated donors contained only 10-20% as many CFU 6 weeks after implantation as did those from non-irradiated donors. In conclusion, 950 rads temporarily decreases the CFU compartment and permanently damages the hematopoietic sites in which they grow. CFU in non-irradiated hematopoietic sites (implants) are stimulated to grow when placed into irradiated hosts, suggesting the presence of a humoral CFU stimulating factor.

96. DIFFERENTIAL EFFECT OF MYLERAN ON HEMATOPOIETIC PRECURSOR CELLS AND THEIR MICROENVIRONMENT IN MICE. L. Delmonte. The differential effect of the alkylating agent Myleran (MY) on femoral hematopoietic precursor cells and their microenvironment was measured in vivo in the differential spleen colony system and in vitro in the agar granulocytic colony system. We studied the total and differential transplantation and retransplantation potential of spleen colony forming units (CFU) and the total regeneration potential of agar colony forming cells (CFC) when MY was administered to the donor CFU and CFC in the presence of the hematopoietic microenvironment, 2) to the donor CFU and CFC in the absence of the hematopoietic microenvironment, and 3) to the primary host hematopoietic microenvironment devoid of CFU and CFC. In intact mice MY depressed CFU 29-fold and CFC 7.4-fold by 24 hrs. The spleen colony erythrocitic:granulocytic (E/G) ratio was increased 2.6 - 3.6-fold. MY directly induced depression of CFU and CFC in the absence of the microenvironment, and altered the ability of CFU to generate CFC. Furthermore, MY selectively eradicated a CFU subpopulation, leaving a surviving CFU population with an increased potential for forming erythrocytic colonies. MY rendered the hematopoietic microenvironment defective for the cloning of CFU and CFC. It depressed the CFU seeding efficiency and the CFC regeneration potential of CFC from the seeded CFU. MY also, by an unknown mechanism, increased the seeded CFUs' potential to form erythrocytic colonies. In all three protocols, MY appeared to affect the CFU in such a way as to select out CFU with an increased potential for developing into erythrocytic colonies upon transplantation from primary into secondary hosts.
Marrow transplantation in canine cyclic neutropenia. 

Cyclic neutropenia is a rare hematological disorder found in humans and the grey collie dog. Previous studies have described the characteristics of the affected grey collie dog in detail, but have not established whether the regular, recurring cycles of neutropenia are due to a stem cell defect or to a marrow microenvironment defect. Transplantation of marrow from an affected grey collie into a normal littermate was undertaken to clarify this point.

Five cycles of neutropenia and reticulocytopenia were observed in a single grey collie dog, while his clinically healthy littermate showed no consistent variation of total neutrophil or reticulocyte counts. The dogs were of opposite sex and genotypically identical in the major histocompatibility loci defined serologically and by mixed leukocyte culture. Following 1200 R total body irradiation, the normal littermate received $5.1 \times 10^9$ bone marrow cells from the grey collie. No post-transplant immunosuppression was used. Regular cycles of neutropenia were clearly evident in the recipient after recovery from radiation induced pancytopenia. Cytogenetic studies confirmed the presence of only donor marrow cells. The occurrence of cyclic neutropenia in the previously normal recipient of marrow from a grey collie donor proved that the defect responsible for this phenomenon is intrinsic to the marrow stem cell, and not dependent on the host environment.

Colony forming unit suicide in Rauscher leukemic mice given tritiated thymidine in vivo. 

We have previously reported that Rauscher viral leukemia results in an increase in the number of hematopoietic colony forming units (CFU) in the spleens of SJL/J mice. This increase parallels a rapid exponential increase in total spleen size in the leukemic mouse, reaching as high as 6–8 times normal by the 14th day and 28 times normal by the 30th day after virus administration. In order to determine the effect of this leukemia on the number of CFU in cycle or capable of being recruited into cycle we have injected the leukemic mice with large doses of tritiated thymidine during the exponential phase of spleen growth. Both short (1 hr.) and long (24 hr., repeated injections) term $^3T$ exposures were utilized in a manner similar to that described by Bruce and Meeker. It was found under both conditions that the proportion of CFU in the leukemic mouse killed by $^3T$-thymidine "suicide" was equivalent to that killed in the normal mouse. These results are in sharp contrast with those previously reported for L1210 leukemia, where more CFU were killed by $^3T$-thymidine in the leukemic mouse than in the normal. The results also show a significant feed-in of Rauscher leukemic CFU into the non-cycling state, such that by 14 days there are at least 400,000 CFU in that state in the leukemic spleen compared to the normal mouse where all of the splenic CFU (both cycling and non-cycling) total approximately 80,000.

Cinemicrographic studies on the growth of granulocytic colonies in mouse bone marrow cultures. 

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GRANULOCYTE COLONIES DERIVED FROM LYMPHOCYTE FRACTIONS OF NORMAL HUMAN PERIPHERAL BLOOD.

Zucker-Franklin, D., Grusky, G. and L'Esperance, P. NYH Sch. of Med. and Sloan Kettering Inst., New York. Normal human peripheral blood contains precursor cells which can give rise to granulocyte and monocyte colonies in vitro. The identity of these cells and the degree of maturation achieved remain unknown. Studies were undertaken to 1) assess the ability of purified lymphocyte fractions to generate various types of colonies, and 2) to develop a method to study entire colonies and their component cells ultrastructurally. "Mononuclear" fractions prepared by Ficoll-Hyphaque gradients were further purified by magnetic removal of monocytes following incubation with iron filings. The preparations contained <3% phagocytes and were free of granulocytes. Cultures were set up according to Pike and Robinson (J. Cell Physiol 76:77, '70). Fixation at 5, 9, 15 and 21 days by addition of 3% glutal took place for 12-15 hours at 37°C. Subsequent procedures were conducted so that the entire agar dish became a "block" with undisturbed colonies. Each colony could be cut out and thin sectioned. At 5 days colonies consisted of undifferentiated, a-granular cells, with irregular shape, large nucleo-cytoplasmic ratio and abundant ER. No small lymphocytes were seen. At 9 days, some colonies had differentiated into the granulocytic series. Promyelocytes were distinguished by large peroxidase-positive primary lysosomes which formed on the concave aspect of the Golgi. Myelocytes were identified by specific granules which emerged from the convex region of the Golgi. Eosinophil granules had a typical osmophilic core and a less dense, peroxidase-positive matrix. Huge cells whose cytoplasm was replete with grey inclusions were tentatively identified as macrophages. Thus stem cells able to give rise to granulocytes appear to be present among purified lymphocyte fractions of normal human blood.

AUTOMATED CYTOCHEMISTRY MONITORING IN ACUTE LEUKEMIAS

M. A. Atamer, W. Groner*, P. O. Sobol*
Hematology Research Laboratory, Grasslands Hospital, Valhalla, New York

It is increasingly essential to combine cytochemical analysis with morphological studies in Clinical Hematology. Chronic granulocytic leukemia is one example in which the combined approach has provided more accurate diagnosis. Recently, methods have been developed to identify cells automatically and correlate the results with microscopic evaluation of Romanowsky stained cells. The automated differential white cell count used in this study utilizes cytochemistry in distinguishing leukocytes. Peroxidase activity (at pH 3.5) differentiates myelocytes, eosinophils, and peroxidase-negative cells. Monocytes are identified by lipase activity and the unstained lymphocytes are selected by light scattering. Correlation between these procedures and Romanowsky staining for normal population has been previously reported.

In this paper, the results obtained from three cases of adult leukemia (2 granulocytic and 1 monocytic) are reported. The patients received similar therapy consisting of hydroxyurea followed by cytosine arabinoside and thioguanine. The entire course of these patients was investigated using both automated cytochemistry and morphological criteria to provide the differential leukocyte count. The results were compared for consistency of classification, precision, and to determine the transformation matrix relating the categorization of the bizarre cell types (blasts and primitive forms). It is demonstrated that the automated cytochemical method is a useful adjunct to morphological examination in monitoring leukemias. It provided rapid (approximately 1 min) accurate (2-10 cell/mm3) counts even in the extreme leukopenia encountered during chemotherapy. Further, the enzyme chemistry is helpful in appropriately classifying the leukemic cells.

PRODUCTION OF COLONY STIMULATING FACTOR IN MITOGEN STIMULATED LYMPHOCYTE CULTURES

Parker, J. W.* and Metcalf, D.* Univ. So. Calif. Sch. Medicine, Los Angeles and Walter and Eliza Hall Institute. We have previously reported that colony stimulating factor (CSF), the glycoprotein regulator of granulocyte/macrophage proliferation and differentiation in vitro, is produced in mixed leucocyte cultures (MLC) and by spleen cells from mice undergoing a graft-vs-host reaction (8th Leucocyte Culture Conference, Upsala). The granulocyte/macrophage system may be activated by the production of CSF in vivo by proliferating lymphocytes responding to antigenic stimulation. Since unfractuated spleen and lymph node suspensions were cultured it was possible that responding lymphocytes produced a factor which stimulated macrophages to produce CSF. We have stimulated leucocyte cultures with phytohemagglutinin-M (PHA), pokeweed mitogen (PWM), cononavalin A, (conA) and bacterial lipo-polysaccharides (LPS). "Purified" lymphocyte suspensions have been prepared and stimulated with PHA and PWM. Media from these cultures were assayed for CSF by the agar culture technique described by Metcalf. All 4 mitogens stimulated CSF production after a delay of 1 to 2 days in culture. CSF activity correlated with 3H thymidine incorporation at 3 days. LPS stimulated spleen cells from congenitally athymic mice to produce CSF, but PWM did not. Removal of adherent cells did not significantly reduce the production of CSF. Both adherent lymphocytes to PHA, but markedly decreased stimulation by PWM. Spleenic macrophages in petri dishes were not stimulated to produce CSF with PHA or PWM or conditioned media from MLC's. We conclude that lymphocytes stimulated to transform and proliferate can synthesize CSF. It appears that both thymic-dependent and -independent lymphocytes may be involved.
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103.
SEX DIFFERENCES IN NORMAL HUMAN GRANULOPOIESIS IN VITRO. Rosenblum, A.L.,* Bull, J.M.,* Mabry, R.J.,* and Carbone, P.P. NIH, National Cancer Institute, Bethesda, Maryland. Marked erythropoietic differences between the normal adult male and female are well documented. Heretofore, a male-female difference in granulopoiesis has not been clearly demonstrated. Utilizing a semi-solid in vitro culture technique the granulocyte colony formation (CFC) was tested using non-fractionated marrow aspirates of 16 normal adult males and 10 normal adult premenopausal females. A dose response curve for all subjects was examined. The slopes of these curves were analyzed by determining the change in colony number between 1.0x10^5 cells/plate and 2.0x10^5 cells/plate. The steepness of the slope correlates with the efficiency of marrow granulocyte colony formation. The slopes of the male-female dose response curves were found to differ significantly. The male slope was 51±4.9 colonies/10^5 cells vs. the female slope of 2x4.6 colonies/10^5 cells (p<0.1). The male CFC was mean 46.4±3.3 colonies/10^5 cells plated compared to the female mean of 28.7±3.4 colonies/10^5 cells plated (p<0.01). When an androgen preparation (testosterone propionate 10^-8M) was added to 4 marrow cultures the marrow CFC increased to mean 29% (range 18-54%) above the control value. These findings demonstrate that normal male marrow has a greater granulocyte proliferative capacity than female marrow. Granulopoietic activity is increased in vitro in the presence of exogenous androgen. These results suggest a hormonal influence on normal human granulopoiesis that has not been previously appreciated.

104.
DRUG INDUCED AGRANULOCYTOSIS: STUDY BY IN VITRO CULTURE TECHNIQUE. Shadduck, R.K., Univ. of Pitts., School of Med., Montefiore Hosp., Pitts., Pa. Patients with drug induced agranulocytosis were studied for bone marrow content of granulocytic colony forming cells (CFC), serum levels of colony stimulating factor (CSF), capacity of peripheral leukocytes to elaborate CSF and evidence for antibody formation. At the time of study, 5 patients had leukocyte counts of 400-1800/μl and 0-150 granulocytes/μl; 4 were followed to recovery. Autonomic colony formation was decreased in 2 patients, 0-7 colonies/2 x 10^8 marrow cells, and normal in a third patient, 62±5. Marrow cells from all three of these patients showed normal colony formation (19-89 colonies) when plated with leukocyte derived CSF. A repeat study on one patient was done during the recovery phase. Serum CSF was increased in 2 of 4 patients (6-19 colonies/0.1 ml) in the neutropenic phase and returned to undetectable levels in one patient followed to recovery. Leukocyte conditioned media generated 2-30 colonies/0.1 ml; highest activity was observed in association with increased monocyte levels during recovery in one patient. A modest reduction in normal CFC's was observed by addition of marrow serum from one patient (6±5 to 30±2) and from another (45±1 to 30±1); other suspected agents did not limit colony formation. These results show that, during the neutropenic phase, patients with drug induced agranulocytosis have normal numbers of granulocytic stem cells and in 2 of 4 patients, elevated serum CSF. In several cases, myelosuppression may have resulted from a drug-antibody interaction. Where multiple drugs are involved, studies by this technique may prove useful in the identification of the offending agent and should offer further information regarding the etiology of this disorder.

105.
MODULATION OF MURINE WBC PROLIFERATION IN DIFFUSION CHAMBERS. Marmor, J.B.,* Russell, J.L.,* and Robinson, S.H. Beth Israel Hospital, Boston, Mass. WBC proliferation in Millipore diffusion chambers (DCs) as a function of cell concentration and time reveals patterns suggesting modulation of stem cell proliferation. DCs were charged with different numbers of normal mouse marrow cells and implanted in mice given 500R 24 hours earlier. Cell number regularly fell initially, then increased logarithmically from day 3-7. Following day 7 cell number remained at a stable plateau until studies were terminated at 14-21 days. The height of this plateau was reproducibly 15-20 times cell input over a range of inputs from 1x10^5-1x10^6 cells/chamber. This proportionality was not observed with higher inputs all of which yielded similar plateaus of about 2.5x10^6 cells/chamber, presumably because of high cell density. Another mechanism must explain the lower, proportionate plateaus found with lower cell inputs. Additional studies showed this to be intrinsic to the cells in DCs and not related to changes in the host mice or chambers. Identical logarithmic growth curves were obtained with fresh marrow cultured in mice irradiated 8 rather than 1 day earlier. Conversely, no additional rise in cell number was observed with cells harvested from DCs at day 7 and recultured in fresh DCs in freshly irradiated mice. Although DCs contain predominantly mature granulocytes after day 7, there are also early WBCs present which take up 3H-Tdr and undergo mitosis; hence there appears to be a steady-state of cell renewal in the plateau period. Moreover, numerous agar CFUs are recoverable from DCs during this time. These findings suggest two possible interpretations: that the cells which proliferate in DCs are capable of only a restricted number of divisions; or, more probably, that cell proliferation is modulated within DCs as mature cells develop, perhaps by cell-cell interactions.
This work deals with the genetic distinction between the viral agents involved in human acute leukemia with high blast cytokinetic characterization of human acute leukemia before and after treatment, Sarna, G.*,

DNA synthesized endogenously from reverse transcriptase reaction contain among the hybridizable nucleotide sequences 50% which cannot hybridize to RNA from the homologous leukemia virus genome. Contrast, DNA synthesized by disrupted particles from human acute leukemic myeloblasts hybridizes 4 to 5 times better to RNA from animal sarcoma-leukemia virus complexes than from the pure leukemia viruses (Proc. Nat. Acad. Sci., USA, in press) The human leukemic particles, therefore, synthesizes viral-related, sarcoma-specific DNA synthesized by human acute leukemic cells. Gillespie*, D. Saxinger*, W.C. Miller*, N. Gallagher*, R.E., and Gallo, R.C. Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. This work deals with the genetic distinction between the viral agents involved in human sarcomas and leukemias. The viral agents was in animals (birds, cats, and mice) appear to be different. Animal RNA tumor viruses of the sarcoma-leukemia class contain genetic information ("sarcoma-specific") which is lacking in the purely leukemia virus population. DNA molecules synthesized endogenously by disrupted mouse sarcoma-leukemia virions (50% sarcoma virus, 50% leukemia virus) through a reverse transcriptase reaction contain among the hybridizable nucleotide sequences 50% which cannot hybridize to RNA from the homologous leukemia virus genome. Contrast, DNA synthesized by disrupted leukemia virus particles anneals equally to RNA from the sarcoma-leukemia virus complex or from the sarcoma-free leukemia viruses. DNA synthesized endogenously from reverse transcriptase of disrupted particles from human acute leukemic myeloblasts hybridizes 4 to 5 times better to RNA from animal sarcoma-leukemia virus complexes than from the pure leukemia viruses (Proc. Nat. Acad. Sci., USA, in press). The human leukemic particles, therefore, synthesizes viral-related DNA which is operationally defined as "sarcoma-specific", even though the clinical diagnosis of the disease is leukemia. These results and other properties of the viral-like human leukemic particle indicate that the human agent may be a defective sarcoma RNA virus and give biochemical cause for questioning an etiologic distinction of human sarcomas and leukemias.

Cytokinetic Characterization of Human Acute Leukemia Before and After Treatment, Sarna, G.*,

Peripheral white cells from patients with acute leukemia with high blast counts were labeled in vitro with tritiated thymidine (3HtdR) and then separated on the basis of size in a lg sucrose gradient sedimentation chamber. Two of these patients were studied prior to and sequentially during chemotherapy. Results of these studies suggest that 1) blasts from patients with acute lymphoblastic leukemia (ALL) were smaller and had less variability in size than blasts from patients with acute myelogenous leukemia (AML), 2) 3HtdR uptake was confined to the large cell fractions in all cases, 3) in a patient with ALL, therapy with L-asparaginase caused a rapid diminution of the total blast population with preferential loss of both large and medium sized cells, leaving a small cell population with low 3HtdR labeling. The data are consistent with a drug induced G1/S block decreasing recruitment + destruction of cells in S phase 4) in a patient with AML, therapy with cytosine arabinoside and 6-thioguanine resulted in an early shift of the blast distribution to larger cells with increased 3HtdR labeling followed by a rapid diminution of all cells with a preferential loss of large cells. This suggests recruitment of cells into cycle as well as destruction of S phase cells. On relapse cytokinetic analysis revealed a narrow distribution of small cells with low labeling activity. The data support a cytokinetic model of advanced human acute leukemia in which, within a given population of blasts, cell size correlates with stage in cell cycle changes in cell size distribution may reflect drug effect and thus be useful in evaluating therapeutic efficacy and for coordinating sequencing of multiple drug therapy. Degree of diminution of the small cell compartments may reflect recruitment and destruction of resting cells.
109.
REMISSION IN MYELOBLASTIC LEUKEMIA: CLONAL SUPPRESSION OR MATURATION? Craddock, C.G. and Crandall, B.F.* U.C.L.A. Medical Center, Los Angeles, California. Serial chromosomal analyses and studies of marrow in culture were done in an elderly patient with myeloblastic leukemia carrying a hyperdiploid C-group chromosome. Remission ensued following non-ablative measures including vincristin, prednisone, 6-thioguanine, leukocyte transfusion and androgen therapy. Remission began while the hyperdiploid line was abundantly present. Behavior of the marrow in culture (colony-formation in soft agar and emergence of lysozyme producing cells after 1 month in culture) continued to be abnormal after remission was achieved. After 185 days in remission cytogenetic and cultural behavior of the marrow were normal although a small percentage of morphologically abnormal cells remain in marrow and blood. Antimicrobial function of the patient's granulocytes was normal at this point. The findings point to resumption of cellular differentiation to a more normal level by myeloblastic tissue rather than to elimination or suppression by leukemic tissue as the mechanism of improved hematopoiesis in this instance. This supports recent in vitro observations indicating that leukemic myeloblasts may mature in culture.

110.
MECHANISMS OF RESISTANCE TO 6-THIOPURINES IN HUMAN LEUKEMIA. Rosman, M., Lee, M.H. and Sartorelli, A.C. (intr. by Skeel, R.) Departments of Medicine and Pharmacology, Yale University School of Medicine, New Haven, Connecticut. The biochemical mechanisms by which leukemic leukocytes of man acquire insensitivity to 6-thiopurines are unknown. These purine antimetabolites are activated to tumor-inhibitory forms by conversion to the nucleotide level. The activities of anabolic and catabolic enzymes involved in the formation and metabolism of analog nucleotide were assayed in leukocytes of leukemic patients considered either susceptible or resistant to these agents. Altered ratios of the activities of hypoxanthine-guanine phosphoribosyltransferase (H-GPRT) and adenine phosphoribosyltransferase (APRT) were noted in 3 of 11 resistant AML and in 1 of 7 resistant ALL patients. One of the resistant AML patients had a marked decrease in H-GPRT activity, while the remainder were relatively deficient, as defined by an APRT/H-GPRT (A/H) ratio greater than the average ratio in untreated patients plus two S.D. units. Particulate-bound alkaline phosphatase was significantly elevated in 4 of 11 resistant AML and 6 of 7 resistant ALL patients. Little correlation between acid phosphatase levels and clinical status was noted. Increased alkaline phosphatase activity tended to occur in resistant patients with normal A/H ratios. The findings suggested that either a decrease in H-GPRT or an increase in alkaline phosphatase activities were responsible, at least in part, for insensitivity to 6-thiopurines in 6 of 11 AML and 6 of 7 ALL patients.

111.
TRANSPORT AND ENZYMATIC MODELS FOR METHOTREXATE RESISTANCE. F.M. Huennekens, J.I. Rader*, G.B. Henderson*, V.G. Neef*, R.C. Jackson* and D. Niethammer*. Department of Biochemistry, Scripps Clinic & Research Foundation, La Jolla, California. Methotrexate (MTX) resistance in human leukemic cells has been attributed to: (a) an increase in the intracellular target enzyme, dihydrofolate reductase; and (b) decreased transport of the drug into the cells. These putative mechanisms of resistance can be studied conveniently in cultured Li210 mouse leukemia cells. In these cells, two separate systems exist for the transport of folate compounds: one specific for folate, and the other specific for 5-methyl tetrahydrofolate and MTX. This has been established by the following lines of evidence: (a) substrate competition (Nahas and Bertino); (b) sensitivity to mercurials; and (c) transport mutants. Iodoacetate also impairs MTX influx but, unlike mercurials, it increases the steady-state, intracellular level of the drug. The latter consists of two components: (a) exchangeable MTX; and (b) MTX bound to dihydrofolate reductase. Increased levels of dihydrofolate reductase that result from treatment of cells with MTX have been shown previously to be due to an increased stability of the enzyme containing bound MTX. An enzyme-MTX-TPNH complex has now been isolated from Li210 cells treated with MTX. Other binary and ternary complexes of the enzyme with substrates and inhibitors have been prepared synthetically and characterized by absorbance spectra and gel electrophoresis. These transport and enzymatic studies have helped to interpret observations made during exposure of Li210 cells to progressively higher concentrations of MTX. Resistant mutants are characterized first by moderate elevations in the level of dihydrofolate reductase. This is followed by the appearance of transport mutants and, finally, by cells having extremely high enzyme levels.
MECHANISM OF ACTION OF VINCRIStINE: SELECTIVE KILLING OF STATIONARY CELL POPULATIONS.
Rosner, F., Grunwald, H., and Hirshaut, Y*. Queens Hospital Center Affiliation of the Long Island Jewish-Hillside Medical Center, the Sloan-Kettering Institute for Cancer Research and the State University of New York College of Medicine at Stony Brook.

A method for evaluating the effects of cytotoxic drugs, both singly and in combination, using human lymphoid cells grown in long-term tissue culture has been previously reported. By this technique, the addition of prednisolone to vincristine has been shown to augment the cell kill achieved by vincristine used alone, at concentrations as low as 2.6ng/ml of vincristine and 800mg/ml of prednisolone. Drug levels considerably higher than these are achieved in patients with current treatment protocols. The potentiating effects of drug combinations in vitro cannot be consistently shown if the drugs are added without attention to the growth rate of the cultured population. If the cultured cells have achieved maximum density and have remained at this density for more than 24 hours they are readily killed by vincristine alone and no further kill is achieved by adding prednisolone. Rapidly growing cultures, however, are relatively resistant to vincristine. The addition of prednisolone to such cultures restores the vincristine susceptibility. Long-term tissue culture is no more effective than vincristine alone in stable populations. Studies with 'H-thymidine show that populations with the lowest labeling index are most sensitive to vincristine. These findings indicate that the cytotoxicity of vincristine cannot all be explained by its effect on the mitotic spindle; it may also block the transfer of cells from G0 to G1. Steroids have been found to slow the transition from G0 to S phase. If more cells remain in G0 the number entering G1 is likely to increase. Vincristine's major kill may then be achieved by trapping cells in G0.

Prolonged survival of Friend leukemic mice following splenic autoimplantation.
Levy, S.B., Tavassoli, M., and Friend, C. New Eng. Med. Ctr., Boston, Scripps Clinic, La Jolla, Mt. Sinai Med. Sch., CUNY, New York. Friend virus induced erythroblastic leukemia is a fulminant disease in mice heralded by short latent period, rapid splenomegaly and an average survival time in susceptible DBA/2J or Swiss mice of approx. 40 days. Since the spleen is the first organ of marked leukemic involvement experiments were designed to study the relationship of this organ to Friend leukemia and host resistance. The involved spleens were removed from leukemic mice, sections taken, and tissue pieces implanted in the same animal. The implanted spleen showed signs of regeneration and the autoimplanted leukemic animals lived longer than intact or splenectomized leukemic controls. A small percent of these autoimplanted animals have lived longer than 400 days without leukemia, whereas all the untreated controls have died. In non-surviving animals the autoimplanted spleen eventually became leukemic; in long-term survivors the spleen implant appeared normal. Occasionally, however, small foci of Friend leukemia could be seen histologically in the implant while the animal showed no evidence of overt disease. Normal spleen implants placed into splenectomized mouse enter G0 and 800ng/ml of prednisolone. Drug levels considerably higher than these are achieved in patients with current treatment protocols. The potentiating effects of drug combinations in vitro cannot be consistently shown if the drugs are added without attention to the growth rate of the cultured population. If the cultured cells have achieved maximum density and have remained at this density for more than 24 hours they are readily killed by vincristine alone and no further kill is achieved by adding prednisolone. Rapidly growing cultures, however, are relatively resistant to vincristine. The addition of prednisolone to such cultures restores the vincristine susceptibility. Long-term tissue culture is no more effective than vincristine alone in stable populations. Studies with 'H-thymidine show that populations with the lowest labeling index are most sensitive to vincristine. These findings indicate that the cytotoxicity of vincristine cannot all be explained by its effect on the mitotic spindle; it may also block the transfer of cells from Go to G1. Steroids have been found to slow the transition from G0 to S phase. If more cells remain in G0 the number entering G1 is likely to increase. Vincristine's major kill may then be achieved by trapping cells in G0.

MARROW TRANSPLANTATION FOLLOWING CYCLOPHOSPHAMIDE (CY) AND TOTAL BODY IRRADIATION (TBI) FOR THE TREATMENT OF ACUTE LEUKEMIA. Buckner, C.D., Cliff, R.A., Fefer, A., Johnson, L., Lerner, K.G., Neiman, P.E., Storb, R. and Thomas, E.D. University of Washington School of Medicine and the U.S. Public Health Service Hospital, Seattle, Washington. The purpose of this study was to evaluate the results of marrow transplantation following high doses of CY and supralethal TBI in patients with acute leukemia who were refractory to chemotherapy. Seventeen patients, ages 9-21, had acute lymphocytic leukemia (ALL) and 15, ages 10-51, had acute myelocytic leukemia (AML). CY, 60 mg/kg, was administered on each of 2 successive evenings followed in 3-5 days by 1000 rads of TBI administered at a dose rate of 5.5 R/min. from dual 60Co sources. Marrow from an HL-A matched sibling, negative in mixed leukocyte culture, was infused within 24 hours after TBI. Methotrexate (MTX) was administered on days 1,3,6, and 11 and weekly thereafter for 100 days to prevent or ameliorate graft-versus-host disease (GVHD). Antithymocyte globulin (ATG) was administered to 13 patients with severe GVHD. Thirty-one of the 32 patients showed evidence of engraftment. Four patients died in the first 3 weeks of infection. Four patients (3 ALL, 1 AML) died between 51 and 94 days with either residual or recurrent leukemia. Twenty-one of the 32 patients had GVHD. Fifteen of the 21 died between 22 and 107 days of infections probably related to the immunosuppression associated with GVHD and its therapy, most frequently of interstitial pneumonitis presumed to be of viral etiology. Four patients with ALL and 4 with AML are alive and free of disease 4, 6, 7, 8, 11, 14, and 18 months after grafting. The long and continuing disease-free interval without further therapy in one quarter of those refractory patients is encouraging.
115.

INFECTION PREVENTION IN ACUTE NONLYMPHOCYTIC LEUKEMIA WITH CONSEQUENT IMPROVED REMISSION RATES AND SURVIVAL DURATION. Schimpf, S.C., Greene, W.H., Young, V.M., Fortner, C.L., Jepsen, L., Cukack, N.E., Wierink, P.H., NCI-Baltimore Cancer Research Center, Baltimore, Md. From 7/1/69 to 6/30/73, 60 patients were prospectively randomized to either I) reverse isolation in laminar air flow (LAF) rooms utilizing cooked food plus sterile water and supplies in addition to oral nonabsorbable antibiotics (gentamicin 250 mg q 4h, vancomycin 500 mg q 4h, nystatin tablets 4 million units q 4h, and nystatin liquid 1 million units q 6h - GVN) or to II) regular hospital ward care plus the GVN regimen or to III) regular hospital ward care. All patients were noninfected adults with acute nonlymphocytic leukemia admitted for their first induction or first relapse. Medical, nursing and antileukemic therapy were comparable in each group as was the extent of granulocytopenia. Regimens I and II markedly reduced patient colonization by exogenous pathogens and substantially reduced the patients’ endogenous flora. Patients in groups I or II had fewer infectious episodes (30, 30) compared to group III (60 infections). Septicemias occurred in 27% of group I patients but in 48% of group III. Pneumonias, rectal abscesses and urinary tract infections were similarly reduced as were colonization and infection by Pseudomonas aeruginosa. Early infectious deaths occurred in 13%, 22%, and 29% respectively of groups I, II and III. Complete remissions occurred in 36%, 67%, and 24% of groups I, II, and III. Increased remission rates in I and II correlated with significantly increased survival compared to the routine hospital care of group III. The regimens in groups I and II have substantially reduced infection and infectious death with an associated improvement in complete remissions and ultimate survival.

116.

ADRIAMYCIN FOR INDUCTION OF REMISSION IN ACUTE LEUKEMIA IN CHILDREN. Starling, K.A.; Berry, D.H.; Britton, H.A.; Humphrey, G.B.; Vats, T,, and Ragab, A.H. Baylor Coll. of Med., Univ. of Arkansas Med. Ctr., Univ. of Texas Med. Sch., San Antonio, Univ. of Okla. Med. Ctr., Univ. of Kansas Med. Ctr., and Washington Univ. Sch. of Med. (Southwest Oncology Group) Earlier studies demonstrated the efficacy of adriamycin for induction of remission in children with acute leukemia. The present study compares the efficacy of remission induction and toxicity of 3 treatment regimens utilizing adriamycin alone. Children under 15 years of age with acute lymphatic leukemia (ALL) and acute myelogenous leukemia (AML), who were in relapse and were resistant to all conventional agents for induction of remission, were eligible for the study. Evaluation for response was on day 21. Thirty-one patients were given adriamycin, 15 mg/m2 q 6h x 6. Three achieved complete remission (CR), 2 achieved marrow remission (M1PR), 10 achieved partial remission (PR), and 16 failed to respond (NR). Twenty-seven patients were given 30 mg/m2/day for 3 days. Five achieved CR, 2 M1PR, 5 PR, and 15 NR. Twenty-seven patients were given 90 mg/m2 in a single dose. Three achieved CR, 3 M1PR, 5 PR, and 17 failed to respond. Four of 18 patients who achieved marrow remission in the 3 treatment groups were children with AML. Fourteen children had severe toxicity in the first group, 9 in the second, and 14 in the third. At this time, there is no statistically significant difference between any of the treatment arms, although 30 mg/m2/day appears to be associated with considerably less toxicity and has the best CR + M1PR rate for remission induction. Adriamycin is an effective agent for remission induction in both ALL and AML, and combination drug studies are in progress.

117.

OPTIMAL USE OF ASPARAGINASE IN ACUTE LYMPHOCYTIC LEUKEMIA OF CHILDHOOD. Jones, Barbara and Holland, James F. for Acute Leukemia Group B. The role of Asparaginase (Asn’ase) in induction treatment of acute lymphocytic leukemia (ALL) in previously untreated children has been controversial. This is because of added vincristine (VCR) toxicity, Asn’ase toxicity per se, and uncertain contribution to remission frequency and remission duration. Acute Leukemia Group B (ALGB) study 6801 showed no significant contribution of a five day infusion of Asn’ase before vincristine (VCR) and prednisone to overall remission frequencies or duration. ALGB study 7111 prescribed Asn’ase 1,000 U/kg/day IV for 10 days, before, during, or after a 3 week VCR and steroid induction, or no Asn’ase. In the 455 patients in the control and the three Asn’ase treatment regimens no difference in remission frequency occurred (82-86%). Median durations of remissions on composite multi-drug maintenance regimens were analyzed for the effect of Asn’ase: without M=13 months, before M=16 months, with M=16 months, after median not yet reached at 20 months. Mortality during the induction phase was 2.5% in VCR and steroid alone, and 2.4% when Asn’ase was given after VCR and steroid. When Asn’ase was given before VCR and steroid, however, mortality was 8.9% and when simultaneously 10.2%. In each of these latter regimens 3% of the patients were considered to have had mortality directly associated with the drugs. Because of equivalent induction potential, lower mortality and longer subsequent remission duration Asn’ase is superior when given after VCR and steroid induction, before maintenance treatment starts. The Asn’ase effect on hepatic metabolism of VCR might influence the outcome since neurotoxicity is greater in the regimens where Asn’ase is given before or simultaneously with VCR and steroid but not when given after.
THE VALUE OF PHARMACOKINETIC ANALYSIS IN INTRATHecal M ethotrexate T herapy. Bleyer, W.A.*,
Sanstead, J.K.* and Ommaya, A.K.* (intr. by H.R. Gralnick). NIH, Bethesda, Md.

Intrathecal methotrexate (IT MX) is frequently used for the prophylaxis and therapy of leukemia and other neoplasms metastatic to the central nervous system. The response and toxicity of this therapy varies widely from patient to patient, perhaps as a result of individual pharmacokinetic variation. We used the dihydrofolate reductase inhibition method to measure MX concentration in 192 cerebrospinal fluid (CSF) samples from 41 patients receiving 12-15 mg/M2/dose. The half-disappearance time of MX in the CSF ranged from 2 to 3 hrs during the day after injection and from 12 to 18 hrs thereafter. Low CSF MX levels (<<0.05) were observed in patients 1) of small body surface area, 2) receiving prophylactic IT MX therapy, and 3) with short remission durations. Elevated levels (>0.05) were associated with 1) large body surface area, 2) presence of malignant cells in the CSF, and 3) serious neurotoxicity. CSF MX concentrations from 5x10^-7 to 2x10^-6 molar for periods of 72 hrs appear to be optimal and can best be achieved with 0.5 to 2 mg of MX administered intravenicularly every 12 hrs via a subcutaneous reservoir. Individual patient dosage being determined by the patient's CSF antifolate level. Five patients with meningeal leukemia treated in this fashion have achieved remission rapidly, without neurotoxicity, and without relapse for periods of 1-11 months to date.

EFFECT OF ERYTHROPOIETIN (ESF) ON COLONIAL GROWTH OF ERYTHROID PRECURSOR CELLS IN VITRO.

Erythroid precursors, isolated from fetal mouse liver by immune hemolysis, respond to ESF by proliferation and differentiation. A method is reported which permits examination of the progeny of single precursors produced in response to ESF, in a medium consisting of supplemented CMRL 1066 with 15% horse serum, 0.8% methylcellulose, at 37°C in 5% CO2. Colonies are scored after 48 hr by retrieving colonies onto slides or by examination of plates after in situ staining with benzidine. In the presence of ESF the number of erythroid colonies is directly proportional to the number of precursors inoculated between 100 and 6x10^5 cells/ml. Colonies are not found with inocula under 10^5 cells/ml; this suggests that a minimal concentration is required for "conditioning" the medium or optimizing the opportunity for essential but undefined cell interactions. The number of colonies is directly dependent upon the concentration of ESF, with a plateau above 0.17U/ml. At optimal concentrations of ESF the colony forming efficiency (colonies/inoculum x 100) is about 5%. The size of individual colonies also depends upon the concentration of ESF. At 0.17U/ml colonies of 64 cells are observed; at 0.034U/ml 16 cells/colony is maximum. At high concentrations colonies contain mature and immature erythroblasts including precursors; lower concentrations do not sustain replication of precursors. Taken together with previous studies on erythroid cells in culture, these observations suggest that ESF initiates both replication and differentiation of precursors. Both the number of precursors triggered to differentiate and the extent of replication of these cells are dose-dependent effects of the hormone.

120.


It is generally accepted that erythropoietin (E) controls the transformation of stem cells to erythroblasts. However, it has been suggested that E also affects the rate of maturation and proliferation of erythroblasts. We, therefore, examined the in vitro action of E in fluid and semi-solid cultures of bone marrow. Firstly, studies were made on 24 and 48 hour fluid suspension cultures of bone marrows from normal and anemic rabbits. Secondly, studies were made on the number and character of erythroid colonies formed in plasma clot cultures of normal rabbit bone marrow, using a technique modified from that of Stephenson and Axelrod (PNAS 68: 1942, 1971). We established that in long term plasma clot cultures E promoted the continuous emergence of erythroid colonies, presumably from stem cells (In Vitro 8:400, 1973). Two day cultures with and without E were now used to test the action of E on maturing erythroid cells. The following distribution of definitive colonies (four or more identical cells) were found in cultures of five normal rabbit bone marrows:

<table>
<thead>
<tr>
<th>Colonies Orthochromatic Basophilic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without erythropoietin</td>
</tr>
<tr>
<td>With erythropoietin</td>
</tr>
</tbody>
</table>

Since the differences are not statistically significant the data suggest that erythropoietin in both the fluid and in the semi-solid culture of bone marrow has little, if any, effect on the maturation or proliferation of erythroid cells.
HEMOGLOBIN SYNTHESIS AND ERYTHROPOIETIN ACTION IN MATURING RED CELL PRECURSORS. Glass, J.*. Lavodor, L.*, and Robinson, S.H. Beth Israel Hospital, Boston, Mass. Erythroid cell differentiation was studied by measuring aspects of hemoglobin synthesis in erythroid precursors at different stages of maturation. Erythroid cells were obtained from murine spleen after phenylhydrazine-induced hemolysis, the more mature cells were removed with antibody to adult RBCs, and the remaining nucleated cells were separated according to size by the velocity sedimentation technique. The fastest sedimenting fraction consisted of 70-80% pronormoblasts and higher fractions contained progressively more mature cells. 59Fe incorporation into heme and activity of ALA-synthetase, the rate-limiting enzyme in heme synthesis, were readily detectable in the pronormoblast fraction, were highest in cells at the basophilic normoblast stage, and then fell off progressively with increasing cell maturity. Only the pronormoblast fraction demonstrated an increase or maintenance of these functions when cultured with erythropoietin (EP) for 18 hours, during which maturation to basophilic and polychromatophilic normoblasts took place. In all higher fractions activity fell with increasing differentiation in vitro. In every fraction, however, activities were lower when cultured in the absence of EP. Similar results were obtained for leucine-3H incorporation into hemoglobin and globin-specific mRNA content, determined with labeled complementary DNA; however, activity increased more sharply and was better maintained with increasing cell maturation than with the measurements of heme synthesis. These findings reflect differences in the patterns of heme and globin synthesis during erythroid cell maturation. EP has the greatest stimulatory effect on the least mature cells, but appears also to act on cells at different stages of maturation.

ERYTHROPOIETIN (EP)-INDUCED STIMULATION OF HEME SYNTHESIS BY DIMETHYLSULFOXIDE (DMSO)-TREATED FRIEND LEUKEMIC CELLS (FLC). Preiser, H.* and Zanjani, E.D. Mount Sinai School of Medicine of the City University of New York, New York, New York. FLC growing in suspension cultures do not respond to EP; however, these cells undergo erythroid differentiation in the presence of DMSO. The present study demonstrates that these leukemic cells when cultured in the presence of DMSO develop the ability to respond to EP. Cells were cultured in the presence or absence of DMSO (primary cultures-PC). At various times after seeding, the cells were harvested and resuspended at a concentration of 4 x 10^6 cells/ml in NCTC 109 with 20% fetal calf serum (secondary cultures-SC). EP was added to 1/2 of these SC. After 1, 2 or 3 days in SC, the cells were incubated for 4 hours with transferrin-bound 59Fe. The incorporation of iron into heme was determined. Only cells grown with DMSO showed increased heme synthesis when EP was added to the SC. Cells which had been cultured with DMSO for as little as 3 days were responsive to EP. For cells which had been cultured in the presence of DMSO for 4 days, EP exerted its maximal effects at 24 hours after the initiation of SC. Heme synthesis was stimulated by more than 100% at this time. EP effects, although still present and significant, were less on days 2 and 3 of SC. Maximal effects were observed with an EP dose of 0.4 units/ml. Morphologically, there was a greater intensity of benzidine staining in FLC from EP-containing SC. These data suggest that the development of EP-responsiveness in erythroid-committed leukemic cells is dependent upon at least one prior maturational event, and that the hormone may enhance heme synthesis in the already differentiated cells.

IMPAIRED ERYTHROPOIETIN RESPONSE IN ANEMIC PREMATURE INFANTS. Buchanan, G.R.* and Schwartz, A.D. (Intr. by D.G. Nathan). Children's Memorial Hospital and Dept. of Pediatrics, Northwestern Univ. Medical School, Chicago, Illinois. The "physiologic anemia" which normal premature infants develop during the first 3 months of life has been attributed to decreased red cell survival, hemodilution, and bone marrow unresponsiveness. The role of erythropoietin (ESF) was investigated by measuring serum ESF levels by the polycythemic mouse bioassay in a group of 16 normal premature infants with varying degrees of physiologic anemia (hematocrit range from 19 to 33%, mean 26%). Injection of 0.5 ml. of the anemic premature infants' serum into polychromatophilic mice resulted in 72-hour 59Fe incorporation ranging from 0.28 to 0.59% (mean 0.42%) compared to 0.55% for the saline control. No ESF activity was therefore demonstrated in any of the premature infants' sera. A simultaneously assayed group of older children with comparable degrees of anemia had elevated serum levels of ESF (59Fe incorporation ranging from 0.99% to 3%). Mixing experiments revealed no inhibitors to erythropoiesis in several of the infants. The absence of ESF despite a moderately severe anemia suggests that an impaired ESF response may be partially responsible for the anemia. This finding is also consistent with previous observations that premature infants have decreased oxygen requirements and can adequately deliver oxygen to their tissues at hematocrit levels which are lower than those of full term infants and older children. Hence low ESF production in such infants would be appropriate.
ANEMIA II AND REGULATION OF ERYTHROPOIESIS

DNA AND RNA SYNTHESIS IN ERYTHROBLASTS ISOLATED FROM BONE MARROW OF PATIENTS WITH INEFFECTIVE ERYTHROPOIESIS (IE). Lourenco, G. *, Kedes, L. and Schier, S. L. Stanford University School of Medicine and the Veterans Administration Hospital, Palo Alto, California.

Previous studies have demonstrated that in idiopathic ineffective erythropoiesis (IEE) are not fully explained by known production deficiency mechanisms. Because there are morphologic nuclear abnormalities and defective maturation of erythroid precursors in marrow, we explored the hypothesis that disordered nucleic acid metabolism accounted for both the biochemical and morphologic observed abnormalities. Erythroblasts were isolated from human marrow by an albumin density gradient technique. We obtained a cell population consisting of 90-95% polychromatophilic and orthochromic erythroblasts and 5-9% PMN's. We established DNA and RNA synthetic rates in the erythroblasts separated from bone marrow in 11 "controls" and the results are recorded as cpm/10^6 erythroblasts/2 hours. DNA synthesis as measured by incorporation of ^3H thymidine was 252±22.4. RNA synthesis as measured by incorporation of ^3H uridine was 7117±0.2. We studied 8 patients with IIE, 3 diGuglielmo Disease (DD) and 3 classical megaloblastoses (2 folate and 1 vitamin B12 deficiency). In all cases DNA synthesis was normal. The ^3H uridine incorporation values in the 2 cases of IIE were 372 and 395.

The patients with DD had values of 303, 341 and 420. The 3 cases of megaloblastic anemia had normal ^3H uridine incorporation with a mean value of 760±193. Thus, of 8 patients with IIE, 2 (IIE and 3 DD) showed decreased ^3H uridine incorporation. The more exact definition of the RNA defect in these erythroblasts will depend on characterization of RNA content and species of these cells, but the data support the hypothesis that in some cases of IIE and DD, RNA metabolism is distinctly disturbed and, as such, might contribute to the pathophysiologic events observed.

E-AMINOLEVULINIC ACID SYNTHETASE (ALAS) ACTIVITY IN EXPERIMENTAL SIDEROBLASTIC ANEMIA. Tanaka, M. * and Bottomley, S. S. University of Oklahoma Health Sciences Center, Oklahoma City, Okla.

Previous studies have demonstrated that in idiopathic sideroblastic anemia in man bone marrow ALAS activity is decreased which may increase with the addition of pyridoxal-phosphate (PLP). The purpose of the present study was to assess bone marrow ALAS activity and its relationship to blood PLP concentrations in an animal model. Experimental sideroblastic anemia was produced in guinea pigs (Harriss et al. Brit.J.Haemat. 11:99, 1965) by the administration of INH (50 mg/kg/day) and cycloserine (CS) (500 mg/kg/day). ± iron (150 mg) for 5 months. ALAS was quantitated radiochemically. Blood and plasma PLP were assayed by the method of Hines and Love (J.Lab.Clin.Med. 73:343, 1969). The results (mean±SEM) of ALAS activity, expressed as pmoles ^14C-ALA formed/10^6 erythroblasts/30 min., and the PLP data are tabulated below.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animals</th>
<th>Ring Sidero- blasts (Pos/Total)</th>
<th>Mean Hgb (g%)</th>
<th>ALAS (pmoles ^14C-ALA)</th>
<th>Whole Blood PLP (pmol/10^6)</th>
<th>Plasma PLP (ng/ml)</th>
<th>Blood PLP (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>5</td>
<td>0/5</td>
<td>13.5</td>
<td>201±36</td>
<td>181±21</td>
<td>119±13</td>
<td>44±6</td>
</tr>
<tr>
<td>Infomer (Fe)</td>
<td>4</td>
<td>0/4</td>
<td>14.5</td>
<td>212±24</td>
<td>223±12</td>
<td>103±12</td>
<td>36±9</td>
</tr>
<tr>
<td>INH+CS</td>
<td>6</td>
<td>4/6</td>
<td>9.1</td>
<td>74±15</td>
<td>101±10</td>
<td>55±15</td>
<td>27±5</td>
</tr>
<tr>
<td>INH+CS+Fe</td>
<td>4</td>
<td>4/4</td>
<td>7.4</td>
<td>93±4</td>
<td>130±10</td>
<td>24±12</td>
<td>6±4</td>
</tr>
</tbody>
</table>

In 2 animals a decrease in marrow ALAS activity and blood PLP preceded the development of anemia. These studies confirm an experimental sideroblastic anemia in guinea pigs which follows the administration of PLP antagonists. The anemia is associated with low blood PLP levels and diminished bone marrow ALAS activity which is only in part corrected by PLP in vitro. It is proposed that PLP deficiency and/or the pathological iron deposition in erythroid cells alter the ALAS apoenzyme or its synthesis.

SIZE, DNA AND HEMOGLOBIN CONTENT OF STRESS NORMOBLASTS. Haley, J. E. *, Frentz, M. *, Brecher, G. and Bessis, M. *. Department of Clinical Pathology and Laboratory Medicine, University of California, San Francisco, California and Institute of Cellular Pathology, Paris, France. A technique has been developed which permits direct measurement of orthochromatophilic normoblasts during extrusion of their nuclei. Measurements were made with a microspectrophotometer which scans the field, registers absorption and integrates by its own minicomputer: cytoplasmic area, nuclear area, hemoglobin content in the Soret band at 415 m and DNA content in the same individually identified cells after Feulgen staining.

Normoblasts extruding nuclei were compared in marrow of normal rats and rats treated with phenylhydrazine (PH). Hemoglobin content and cytoplasmic size were twice normal in PH marrows but DNA content and nuclear size were the same for both groups. All nuclei being extruded were 2n as determined by identical DNA content of mature leukocytes. The data exclude nucleolysis as occurring in either normal or stress erythropoiesis.
MEMBRANE ELASTICITY AND IN VITRO CAPILLARY FLOW VELOCITY OF NORMAL AND SENESCENT RBC. 

P.L. LaCelle, University of Rochester, Rochester, NY. Erythrocyte flow in capillaries may depend significantly on membrane elasticity, cell surface-capillary wall resistance and the character of the thin lubricating film separating membrane and capillary surface. The relatively rigid senescent RBC would be expected to have abnormal flow characteristics. These studies examined membrane elasticity in terms of unidimensional extensibility of membrane as a function of time and force, determined the erythrocyte flow velocity (VRBC) and derived values of membrane capillary wall resistance (C) and lubricating film thickness (δ) in order to compare capillary flow of senescent to normal RBC. Membrane extensibility was measured by aspiration of a portion of RBC into a 0.5μm micropipette at pressures 0-100mmHg and determination of membrane elongation with time intervals 10-300sec. VRBC was recorded directly for RBC flowing in a 2.8μm glass microcapillary; suspended in a 2μM Tris-NaCl isotonic buffer containing 0.25% albumin and γ-globulin concentrations 0-10g% at pressure differentials 0-3mm Hg. The extension of senescent cells increased more rapidly than control cells and whereas the extension ratio (Δx/x) for control membrane was linear with force, in senescent cells initial resistance to extension then rapid loss of extensibility occurred and at times >120sec plastic flow of membrane was observed. VR for control was 6-15μm/sec, ½ that of cell free axially flowing fluid, and VR for senescent cells identical; 5-17μm/sec. Addition of globulin did not affect VR or C which was 1×10⁻¹² dyn sec/cm². γ×10⁻¹⁰μm for both control and senescent RBC. These data indicate different elastic properties of senescent cells and predict retardation of flow in critical channels where time dependent extensibility is important. Normal and senescent cells have similar flow characteristics in capillaries >2.8μm and are not affected by protein concentration.

CONGENITAL HEMOLYTIC ANEMIA DUE TO THERMAL SENSITIVITY OF THE ERYTHROCYTE MEMBRANE. 

Zarkowsky, H.S., Mohandas, N.*, Speaker, C.S.* and Shohet, S.B. Washington University Schools of Medicine and Engineering, St. Louis, Mo. and University of California School of Medicine, San Francisco, California. "Mini-microspherocytes," measuring 2-3μ in diameter, and cells with blunt projections or triangular in shape were diagnostic features of the erythrocyte morphology in 3 children, from 2 unrelated families, with a congenital hemolytic anemia. The hemoglobin was 7.5-9 gms%; hematocrit 25-28%; retics 15-25%; splenomegaly was present and one child developed cholelithiasis at age 2½ years. Splenectomy was performed in 2 children and hemoglobin rose to 11 gms% with retics of 5%. Patients' red cells were incubated in autologous heated and unheated plasma at various temperatures, and serial samples were fixed in gluteraldehyde for scanning electronmicroscopy. The cells underwent no morphologic changes during 4°C incubation for 18 hrs. At 25°C short blunt irregularities of the cell surface developed by 17 hrs. Within 3 hrs of exposure to 37°C gross membrane fragmentation or "budding" began. The cells lost intact cellular strands and spheroi bodies. After 17 hrs all cells had become smooth "mini-microspherocytes." The process of membrane budding was accelerated by increasing the temperature of incubation; so that at 44°C the cells began to bud immediately and by 60 min all cells were smooth spheres. Normal cells required exposure to 49°C for budding to occur. The disc gel electrophoretic pattern of membrane protein was normal. The ratio of membrane phospholipid to protein (16) was less than normal (22) due to an apparent increase in protein content. We propose that the thermal sensitivity of an abnormal membrane component results in instability of the membrane at body temperature and in vivo cell budding. We have designated this new hemolytic anemia - congenital pyropoikilocytosis.

A METHOD FOR QUANTITATION OF ERYTHROCYTE COATING SUBSTANCES IN AUTOIMMUNE HEMOLYTIC ANEMIA. 

MacKenzie, M.R. University of California, Davis, California. Autoimmune hemolytic anemia (A.H.A.) is characterized by a positive direct antiglobulin reaction. Although useful in diagnosis, its quantification is difficult and fails to correlate with disease activity. Methods using complement fixation provide quantitative information but are difficult technically. Described herein is a method of radio inhibition assay which can be utilized by large clinical laboratories. A standard curve is constructed using ¹²⁵I labeled protein (IgG, etc.), high titer monospecific antisera and unlabeled proteins. The antisera must precipitate 85% or more of the available radio-labeled protein. Labeled antigen is incubated with the optimum antisera dilution. The samples are centrifuged and counted. Dilutions of standard antigen are incubated with antisera for 1 hour at 37°C prior to the addition of labeled antigen. As little as 2 ng of protein can be detected for assay. A known quantity of lysed RBC's are substituted for the standard antigen. The degree of inhibition of precipitation of labeled antigen by RBC is related to the inhibition of precipitation of labeled antigen by standard unlabeled proteins and the quantity of protein is calculated. RBC's from normals have less than 50 molecules of IgG/cell present. Seven patients with A.H.A. have had from 185 to 1770 molecules of IgG/cell present. Three of these seven patients when treated with prednisone had less than 100 molecules of IgG/cell. A technique has been developed which allows the quantitation of proteins coating RBC from patients with A.H.A., permitting the monitoring of the effects of therapy. The technique is suitable for the large clinical laboratory, thus making it available to the practicing hematologist.
130.

BONE CHANGES IN PNH. Hutcheson, J. K. & Hartmann, R. C. Vanderbilt Univ. Sch. of Med.,
Nashville, TN. Bone changes in common heritable hemolytic anemias, such as sickle cell disease and
thrombocytopenic purpura, are well known. To our knowledge there are no reports of bone changes in the acquired
disorder, PNH. In a large retrospective study we reviewed available bone x-rays of 15 PNH patients,
age 20 to 68. Dorsal spine: Fourteen had flattened vertebral bodies in region D-8 through D-11 with
width/height ratio > 1.2. Types of collapse were "Lincoln Log," wedged and flat. Lumbar spine: 2/6
vertebral compressions & 4/6 suggestive trabecular coarsening. Hands (metacarpals): 2/7 periosteal
reactions; 1/7 widened medullary cavities & thin cortices; 3/7 thin shafts & narrow medullary cavities. Feet:
5/8 phalangeal periosteal reactions. 2/8 narrow medullary cavities & thin shafts of proximal phalanges with
one pathologic fracture. Skull films: 9/9 cranial vault thickening and 4/9 "hair-on-end" appearance.
Other: Aseptic necrosis of the hip in one. Vertebral compression fractures in PNH strikingly resemble those
in sickle cell disease, & the pathophysiology may be similar. Vascular pattern of vertebral bodies will be
discussed regarding susceptibility to repeated thromboses. Digital periosteal new bone formation may also
stem from bone infarcts. Several experienced mild pain and tenderness in these regions. Bone lesions did
not correlate with severity of anemia or hemoglobinuria; skull lesions, however, were probably the result
of marrow hyperplasia. The frequency of vertebral & digital lesions (though often subtle & initially over-
looked) suggests that small vessel thrombosis is probably more frequent in PNH than generally appreciated.
Such bone lesions in adult, acquired disease suggest that similar lesions in certain heritable disorders may
be independent of the rapid bone growth phase of childhood.

131. XI. HEMOSTASIS AND THROMBOSIS III

ENDOTOXIN-INDUCED CLOTTING AND THE GENERALIZED SIMARIZMAN REACTION IN C6 DEFICIENT RABBITS.
The extent of intravascular clotting and frequency of the generalized Shwartzman reaction (GSR) were compared in normal rabbits and C6 deficient rabbits. Animals were given 25 mg
cortisone acetate daily for 4 d. On the second day 125I-fibrinogen was injected intravenously. On the 4th day the animals were infused over 2 h with 200u/kg of E. coli endotoxin. Blood
samples were taken for measurement of fibrinogen radioactivity and concentration, F. VIII, platelets, WBC and PCV. Fibrinogen consumption over 6 h beginning with the endotoxin infusion
was calculated from plasma volume, fractional loss of plasma fibrinogen radioactivity and mean fibrinogen concentration. Mean WBC counts were: controls, 11,500/mm3 before endotoxin and
1,950/mm3 at 6 h; C6 def. animals, 4,700/mm3 before endotoxin and 200/mm3 at 6 hr. Mean
platelet counts were: controls, 609,000/mm3 before endotoxin and 201,000/mm3 at 6 h; C6 def.
animals, 505,000/mm3 before and 185,000/mm3 at 6 h. Mean fibrinogen levels were: controls,
312 mg/kg before endotoxin and 160 mg/kg at 6 h; C6 def. animals, 218 mg/kg before and 201 mg/kg at
6 h. Mean fall in F. VIII was 69% of preinfusion value for controls and 47% for C6 def.
animals. Mean 6 h fibrinogen consumption was 59 mg/kg ± 7 SE for controls and 24 mg/kg ± 3 SE for C6 def. animals. Six of 7 controls developed the GSR compared to 1 of the 7 C6
def. animals. Thus, late components of complement appear to participate in the processes
inducing intravascular coagulation and the GSR in the cortisone-prepared rabbit given endo-
toxin.

132.

RECOMBINATION STUDIES WITH FRACTIONS FROM NORMAL, HEMOPHILIA A, AND VON WILLERBRAND'S DISEASE

PLASMAS PLUS SMALL ACTIVE AHF FRAGMENTS. Cooper, H.A. Hendrick, E.H.*, and Wagner, R.H.*
University of North Carolina School of Medicine, Dept. of Pathology, Chapel Hill, N.C.
We have reported dissociation of canine and bovine antihemophilic factor (AHF, Factor VIII)
with Ca2+ and recombination of the dissociated fragments of normal canine AHF. Recombination of
dissociated normal human and bovine AHF preparations was investigated. Normal human and
bovine AHF preparations were made 0.25M in Ca2+ and chromatographed on agarose A-15m. An insert
high molecular weight protein eluted in the void volume fractions and a small active AHF
fragment eluted later. The small active fragments of both normal human and bovine AHF recom-
binated quantitatively with their respective high molecular weight fraction. The preparative
scheme used for normal human or canine plasma AHF was used to obtain preparations from the
plasma of humans with von Willebrand's disease (vWD) and hemophilia A and from canine hemo-
philic plasma. When these three types of preparations were chromatographed on agarose A-15m,
the void volume fractions had no AHF activity. These three void volume fractions were mixed
with the small active fragment prepared from the normal plasma of their respective species. Chromatography of the mixtures in agarose gel showed that the fractions from the human hemo-
philic plasmas contained a molecule which bound the small active normal fragment, but neither
the fractions from canine hemophilia A plasmas nor the fractions from the human vWD plasma showed evidence for such material. These data demonstrate that patients with hemophilia A
have a carrier protein for AHF that is abnormal or deficient in subjects with vWD and dogs with
sex linked AHF deficiency. Since the vWD plasma studied also lacked the AHF-like antigen, this
information also suggests that the AHF-like antigen is probably the carrier protein of AHF.
VON WILLEBRAND'S FACTOR AND VON WILLEBRAND'S ANTIGEN, INDEPENDENT MOLECULAR ENTITIES.

The plasma factor (von Willebrand's factor, vWF) which confers Ristocetin aggregability to human platelets is decreased or absent in von Willebrand's disease, usually in parallel with the von Willebrand's antigen (Factor VIII-like antigen, vWF-Ag). However, primary binding immunoelectrophoresis and counterimmunoelectrophoresis provide evidence that they are distinct molecular entities. Antisera were produced by immunization of rabbits and goats with vWF-F and vWF-Ag rich molecular exclusion fractions of plasma cryoprecipitates. Insolubilized gamma globulin fractions from these antisera specifically bind both entities. However, they do not do so in a manner compatible with a one molecule hypothesis because: 1) proportionately more vWF-Ag than vWF-F is bound at several concentrations of solid phase antibodies; 2) the ratio of unbound vWF-F to vWF-Ag varies at different concentrations of solid phase antibody; and 3) at appropriate dilutions of selected solid phase antibodies little or no binding of vWF-F is observed, though approximately 50% of vWF-Ag is bound. These observations suggest that von Willebrand's disease is a multimolecular deficiency state.

IMMUNOLOGIC RECOGNITION OF THE FACTOR VIII/von WILLEBRAND FACTOR (F.VIII/vWF) ON THE PLATELET SURFACE. Coller, B.S.*; Hirshchman, R.J.; Kasten, B.L.*; and Oeralnick, H.R. NIH, Bethesda, MD

Despite evidence of abnormal platelet function (long bleeding time, decreased platelet retention, impaired platelet aggregation to ristocetin and mucocutaneous bleeding) von Willebrand's disease (vWD) has been considered a plasma factor deficiency. A purified protein having both F.VIII coagulant activity and vWF platelet retention activity was used to immunize a goat. The resulting antibody neutralized F.VIII coagulant activity, decreased platelet retention of normal blood and blocked ristocetin aggregation of normal platelet rich plasma. This same antibody also acted as an "anti-platelet" antibody in 5 separate immunologic systems: serotonin release, complement fixation, platelet aggregation (at doses higher than those which blocked ristocetin aggregation), immuno-fluorescence and radioimmunoassay.

Specificity of the antibody was confirmed by the 1) ability of plasma samples from normals, vWD patients and hemophiliacs to inhibit with few exceptions the serotonin release in proportion to their F.VIII/vWF antigen content as measured by counter-immunoelectrophoresis, 2) ability of purified F.VIII/vWF to inhibit serotonin release by the immune serum, 3) ability of an eluate (prepared from washed platelets incubated with the immune serum) to decrease platelet retention, block ristocetin aggregation and reduce F.VIII coagulant activity, 4) ability of platelets to compete with purified F.VIII in a radioimmunoassay.

Our hypothesis is that the F.VIII/vWF present on the platelet surface is either deficient or abnormal in vWD and accounts for the apparent abnormalities in platelet function in vitro and in vivo. The ability to correct these abnormalities with plasma or purified F.VIII/vWF may be accounted for by a dynamic equilibrium between the platelet surface and plasma.

RISTOCETIN INDUCED AGGREGATION OF GEL FILTERED PLATELETS. Olson, J. D.*; Fass, D. N.; Bowie, E.J.W. and Mann, K. G. Mayo Clinic, Rochester, Minn. Ristocetin induced platelet aggregation was studied using the platelet rich plasma (PRP) and the gel filtered platelets (GFP) of normal individuals and patients with von Willebrand's disease (vWD). Ristocetin (0.1%) uniformly aggregated the normal GFP; however, the response in PRP was variable. The PRP and GFP of vWD patients did not aggregate in response to ristocetin. While the addition of normal plasma allowed ristocetin response in vWD GFP; these additions did not correct the PRP.

Factor VIII concentrates were gel filtered through 4% Agarose (Bio-Rad) and fractions collected. Factor VIII procoagulant activity eluted in 2 peaks. The first and largest was near the void volume, and the second in the earlier fractions of the second protein peak. The component required for ristocetin aggregation was assayed using vWD GFP. This component eluted with the early factor VIII containing fractions as well as the early fibrinogen containing fractions. Many fractions which contained either factor VIII or fibrinogen had no effect in correction of the abnormal ristocetin response of vWD GFP. Normal GFP, washed once by sedimentation, were not sensitive to ristocetin in the absence of normal plasma or plasma fractions. The washed GFP provide an alternative substrate to vWD GFP for the assay of the ristocetin aggregation component in plasma or plasma fractions. It appears then that normal plasma contains a component which is necessary for ristocetin aggregation, and that this component co-chromatographs with normal GFP. Present chromatographic data suggests the ristocetin aggregation component is not equivalent to factor VIII. In vWD the PRP not only lacks this normal component but also contains another substance which inhibits the normal component when it is added. vWD GFP contain neither the inhibitor, nor the normal component.
A SIMPLIFIED PLATELET FACTOR 3 (PF-3) ASSAY FOR THE RAPID DETECTION OF PLATELET ISOANTIBODIES AND AN ANTIPATELET FACTOR IN ATP AND SLE. Hirschman, R.J., and Gralnick, H.R. NIH, Bethesda, MD. The PF-3 assay (J.C.I. 51:97a, 1972 and Blood. 56: Press). Maximum release of serotonin occurs in this system only after dilution of the serum, suggesting the presence of blocking antibodies. By use of the same assay we have tested sera from patients with presumed immune thrombocytopenic purpura (ITP), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) in an attempt to demonstrate antibodies against platelet antigens. Control sera released 0.8 ± 0.5% serotonin; sera from untransfused patients with ITP released 11 ± 2.0% serotonin; sera from 4 transfused patients with ITP released 22.7 ± 6.1% serotonin; sera from 11 patients with SLE and 4 patients with RA released no serotonin (0.2 ± 0.2%). The release of endogenous serotonin from normal platelets is produced by a factor, presumably antibody, in the serum of patients with ITP and is a reliable way to distinguish that serum from that of patients with other so-called autoimmune states.

ENDOGENOUS SEROTONIN RELEASE FROM PLATELETS, AN "IN VITRO" TEST FOR THE DIAGNOSIS OF ITP. Tejada, F.* and Zieve, P.D. NCI-VA Medical Oncology Branch. Washington, D.C. and Baltimore City Hospitals. Baltimore, Maryland.

The serum of patients transfused with concentrates of platelets often contains HL-A antibodies which release endogenous serotonin from washed heterologous platelets during incubation at 37°C for 30 minmutes (J.C.I. 51:97a, 1972 and Blood. 56: Press). Maximum release of serotonin occurs in this system only after dilution of the serum, suggesting the presence of blocking antibodies. By use of the same assay we have tested sera from patients with presumed immune thrombocytopenic purpura (ITP), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) in an attempt to demonstrate antibodies against platelet antigens. Control sera released 0.8 ± 0.5% serotonin; sera from untransfused patients with ITP released 11 ± 2.0% serotonin; sera from 4 transfused patients with ITP released 22.7 ± 6.1% serotonin; sera from 11 patients with SLE and 4 patients with RA released no serotonin (0.2 ± 0.2%). The release of endogenous serotonin from normal platelets is produced by a factor, presumably antibody, in the serum of patients with ITP and is a reliable way to distinguish that serum from that of patients with other so-called autoimmune states.

A reproducible method for the quantitative determination of antiplatelet antibody has been developed. The amount of IgG on a platelet may be quantitated by the absorption of a known amount of rabbit, anti-human IgG during incubation with platelets. The amount of anti-IgG remaining following this incubation may be assayed by reincubating with sheep erythrocytes coated with human IgG in the presence of complement and determining the resultant percent lysis. This may then be compared to a standard lysis curve derived by the inhibition of the same amount of anti-IgG with known amounts of human IgG. Thus the amount of IgG on the platelet surface may be calculated. The anti-IgG detects all subclasses of IgG with accuracy. Using this method, normal platelets have less than 1 x 10^-9 grams/platelet. Patients with classical ITP have greater than 100 x 10^-9 grams/platelet. Incubation of normal sera with normal platelets fixes less than 1 x 10^-9 grams/platelet; incubation with ITP sera fixes greater than 10 x 10^-9 grams/platelet. As the platelet count normalizes either with therapy or spontaneous remission, the level of antibody returns to the normal range. In one patient, the antibody was no longer detectable following splenectomy. The highest level of antibody was detected in a patient with post-varicella thrombocytopenia.
HEMOSTASIS AND THROMBOSIS III

139.

EFFECT OF PLASMA, STEROIDS AND STEROID PRODUCTS ON ADHESION OF OPSONIZED HUMAN PLATELETS TO HUMAN LEUKOCYTES. S. Karpaklin and M. Verp.* New York Univ. Med. Sch., N.Y. The mechanism of platelet destruction in patients with immune thrombocytopenic purpura was examined by measuring the amount of surface antigen present on the platelets. The surface antigen was detected by the 51Cr release test, which is a sensitive indicator of the presence of a platelet-specific antibody. The results showed that 51Cr release was elevated in patients with ITP, and that the release was inhibited by the addition of normal plasma, but not by the addition of plasma from patients with ITP. This suggests that 51Cr release is a sensitive indicator of the presence of a platelet-specific antibody.

140.

PLATELET PHAGOCYTOSIS BY ITP SPLENIC LEUKOCYTES. McMillan, R., Longmire, R.L., Yelenosky, R.*, Armstrong, C., Tavaszoli, M., and Yam, L.L. Scripps Clinic and Research Foundation, La Jolla, California. An in vitro system has been developed to evaluate the role of the spleen in the pathogenesis of idiopathic thrombocytopenic purpura (ITP). Splenic WBC suspensions from ITP patients were washed 3 times and then resuspended in MEM. Type specific homologous platelets obtained from normal donors were labeled with 51Cr and washed once with autologous plasma. Mixtures containing 4 x 10^6 platelets and 10^5 homologous platelets in 4 ml of 50% fresh donor plasma were incubated for 1 hr with constant mixing. The WBC and platelet-rich fractions were separated by differential centrifugation. The radioactivity of the WBC fraction (phagocytosed platelets) and the platelet fraction were determined and the percent phagocytosis calculated. Platelet phagocytosis by splenic macrophages was demonstrated by electron microscopy. Mean percent phagocytosis (±S.D.) of control spleens was 16.7 ± 5.6 and of ITP spleens was 18.7 ± 5.0. If the platelet phagocytosis results are tabulated according to the clinical steroid response, the results are: complete responders--18.6 ± 3.1, partial responders--38.5 ± 1.7 (p<0.05) and non-responders--50.6 ± 17.8 (p<0.01). These results appear to correlate with the quantity of "foamy macrophages" seen on tissue prints. Platelet phagocytosis by washed ITP leukocytes occurred in the absence of added ITP plasma, suggesting coinfection with an antibody factor (APF) product. These results provide further evidence that the spleen is an important site of both platelet sequestration and APF production. Although steroids could effect either APF production or platelet phagocytosis, present evidence is most consistent with the latter possibility.

141.


PTP is characterized by the development of an isoantibody to transfused platelets and the paradoxical destruction of autologous platelets. Twelve cases have been previously reported; all were females whose platelets following recovery lacked the PLA1 antigen. We are reporting an additional case: a PLA1 negative male, a PLA1 negative female and a second female, whose platelets after recovery possessed the PLA1 antigen. In each patient, both the 51Cr release assay (Aster, R.H., et al, J. Clin. Invest. 42:1199, 1969) and complement (C') fixation test (Schulman, N.R., et al, Prog. Hematol. 4:222, 1964) were used for detecting isoantibodies. Antibody was detected in all 3 patients by the 51Cr release test, but only one reacted by C' fixation. In the patient whose platelets were PLA1 positive, serum obtained during the acute episode was reactive against 4/11 normals by C' fixation and 17/27 by 51Cr release. In all instances where C' was fixed, 51Cr release also occurred, but the reverse was not true. The antibody did not release 51Cr from the patient's platelets but did from those of one of the two donors who had donated her transfusion. Antibody activity disappeared from serum samples three months after the episode of thrombocytopenia. This probably represents the development of an isoantibody of as yet undefined specificity. These observations indicate that PTP is more heterogeneous than previously suspected and is not confined to women and PLA1 negative individuals. C' fixation does not detect the antibody in all cases of PTP. 51Cr release appears to be a more sensitive indicator.
workers who have concluded that the 55 cell is a T-lymphocyte. E-rosette formation and surface Ig bearing lymphocytes. These studies do not allow the Sezary cell to be classified as either a B- or T-lymphocyte, but indicate that whatever its origin it lacks the surface properties of both B- and T-lymphocytes and lacks as well the ability to respond to a variety of mitogenic stimuli. Our studies are at variance with those of other groups who have concluded that the 55 cell is a T-lymphocyte.

Thrombocytopenia disappears when IgG anti-P1A1 antibody, present in previous cases, is catabolized or removed (absorbed) by exchange transfusion. A 45-year-old woman developed lingual and buccal hemorrhagic bullae, purpura and severe thrombocytopenia (1200/mm³) 7 days after transfusion of 3 units of whole blood. She had previous uncomplicated pregnancies and no evidence of lupus, DIC, splenomegaly or PNH. Marrow was cellular with adequate megakaryocytes. Her platelets were P1A1 negative and her serum contained anti-P1A1 and anti-HLA antibodies of multiple specificities. Absorption with anti-IgG removed antibody activity whereas anti-IgG1, anti-IgG2 and anti-IgG4 did not. Antibody activity was quantitated by platelet 51Cr release and by aggregometer. Seven unit plasmapheresis resulted in a rise of platelet count to 7,000 and a decrease of antibody to 36% of initial activity (51Cr release) but the next morning the count was 2,000 and antibody activity was 49%. Nine unit and 6 unit removal over the next 2 days decreased antibody activity to 7%; subsequent daily platelet counts were 17,000, 27,000, 71,000, 156,000. Anti-HLA activity was less affected by plasmapheresis as was anti-P1A1. This case illustrates the following: 1) post-transfusion purpura with anti-P1A1 and anti-HLA antibodies, 2) IgG anti-platelet antibody activity, and 3) effective, uncomplicated therapy with plasmapheresis which circumvented the hazards of exchange transfusions and severe transfusion reactions.

The Sezary Syndrome lymphoid cell: abnormal mitogenic responsiveness and surface properties. Raul Braylán, Daina Variakojis, and Stanley Yachnin Departments of Pathology and Medicine, University of Chicago, Chicago, Illinois

Sezary’s Syndrome (SS) is a variant of mycosis fungoides (MF) associated with erythroderma, in which the peripheral blood is invaded by large numbers of lymphoid cells possessing indent ed and convoluted nuclei. We have studied the blood lymphocytes of 5 patients with SS whose WBC ranged from 20-140 x 10³/mm³ (50-90% lymphoid cells). Non phagocytic lymphoid cells free of erythrocytes and platelets were isolated by nylon column filtration. Such cells from patients with SS showed markedly defective mitogenic response (2[¹⁴C] thymidine incorporation) to a broad dose range of phytohemagglutinin, pokeweed mitogen, concanavalin A, and a rabbit antihuman lymphocyte serum, when compared with normal human lymphocytes. Ficoll-hypaque mononuclear lymphoid cells from patients with SS were analyzed for their ability to form E-rosettes with unsensitized sheep erythrocytes. Three patients studied showed 3%, 10%, and 28% E-rosettes respectively (n=24.8±7.4%). By way of contrast 3 patients with MF having normal peripheral WBC showed normal lymphocyte responses to mitogens as well as normal proportions of E-rosette forming and surface Ig bearing lymphocytes. These studies do not allow the Sezary cell to be classified as either a B- or T-lymphocyte, but indicate no uniformcy in its origin. It lacks the surface properties of both B- and T-lymphocytes and lacks as well the ability to respond to a variety of mitogenic stimuli. Our studies are at variance with those of other workers who have concluded that the SS cell is a T-lymphocyte.

Immunoglobulins on the surface of human and murine lymphocytes - their distribution on the surface and possible role in cell mobility. Ault, K.A.* and Unanue, E.R.*, (intr. by S. Schlossman) Harvard Medical School, Boston, MA. Surface immunoglobulins (Ig) on human peripheral blood lymphocytes (PBL’s) have been studied and compared with those on murine splenic lymphocytes in order to better understand the importance of the capping phenomenon and its relationship to cell mobility. When reacted with fluorescein tagged rabbit anti-human globulin (RAHg), B cells show fluorescence in small discrete spots scattered uniformly over the surface. On warming to 37°, these Ig sites redistribute predominantly into multiple large aggregates. This is in marked contrast to the rapid and organized "capping" of similarly treated murine lymphocytes. PBL’s show Ig sites distributed in small patches but separated by several thousand angstroms, whereas murine lymphocytes show a diffuse continuous distribution over their surface. This difference in pattern of Ig sites may account for the infrequency of capping seen in PBL’s. We have studied whether cell mobility might be involved in cap formation since caps frequently form on the ends of uropods seen on lymphocytes after warming to 37°. Uropod formation is a reflection of translational cell movement. Cells were studied for frequency of uropod formation after incubation of anti-Ig antibody. Lymphocytes of both species showed increase in the frequency of uropod formation after reaction with anti-Ig antibody. This could be inhibited by preventing contact of the cells with a solid substrate and by agents such as cytochalasin B and diisopropylfluorophosphate, fluorophosphate, both of which prevent cell motion but not capping. Thus, lymphocytes can set into motion by interaction with surface Ig, perhaps by natural antigens. In addition, capping seems to involve intrinsic movement on the cell membrane which is separate from cell motility.
Lymphocytes 1025

145. SCANNING ELECTRON MICROSCOPY OF HUMAN LYMPHOCYTE SHEEP ERYTHROCYTE ROSETTES. Pollock, A. *, Douglas, S. D., Bentwich, Z. *, Pu, S. *, de Harven, E. *, Memorial Sloan-Kettering Cancer Center, Mount Sinai School of Medicine, and The Rockefeller University, New York, N. Y. Sheep red blood cell rosettes (SRBC-R), a marker for human T lymphocytes were prepared with normal peripheral blood lymphocytes (PBL), thymocytes, and a cultured T-cell line. EAC rosettes, a marker for some B lymphocytes were prepared with normal PBL. Samples were prepared for scanning electron microscopy (SEM) by aspirating the cell suspension on to a silver membrane as described previously. About half of the lymphocytes in SRBC-R had a smooth surface architecture, the remaining lymphocytes had a moderate number of surface microvilli as has been described for normal T-PBL. In a small proportion of SRBC-Rs the lymphocytes had multiple microvilli and were indistinguishable from normal B-PBL. Studies of SRBC-R formed with thymocytes, and cultured T-cell lines indicate that the surface of many small T-cells developed microvilli during rosette formation. This finding may account for the more complex surface pattern of some T lymphocytes in SRBC-R. Both transmission electron microscopy (TEM) and SEM revealed point to point contact sites between SRBC and T-lymphocytes. In contrast, complement receptor lymphocytes (CRL) had multiple surface microvilli and smooth lymphocytes did not form EAC rosettes. The areas of contact observed by TEM and SEM between EAC and CRL were invariably broad zones of attachment and rarely point contacts.

146. The Immunological Identification of Malignant Lymphoreticular Cells. Shevach, E., Jaffe, E. *, Eleison, R., and Green, I. NIAID and NCI, NIH, Bethesda, Md. The mononuclear cells participating in immune responses are heterogeneous both morphologically and functionally. The bone marrow derived (B) lymphocyte can be identified by the presence of easily detectable surface immunoglobulin and a receptor for antigen-antibody-complement (EAC) complexes. Monocytes and macrophages also bear a receptor for EAC and in addition possess a receptor for red cell-IgG complexes (EA). In man, thymus derived (T) lymphocytes form non-immune rosettes with sheep red blood cells (E). We have examined a number of malignant mononuclear cell populations for the presence of the EAC, EA, and E receptors on suspensions of cells and have adapted the technique to demonstrate the EAC and EA receptors on frozen tissue sections; also, rosetted malignant cells can be cytologically examined on Millipore filters. The malignant cells both in section and suspension from the spleens and lymph nodes of 4 patients with nodular lymphoma bound the EAC reagent and did not bind the EA reagent; by this criteria these malignant cells are of B lymphocyte origin. The malignant cells from the spleens of 2 patients with leukemic reticuloendotheliosis and 1 patient with malignant histiocytosis could be classified as being of monocyte origin by the binding of the EA reagent. In 3 cases of Hodgkin's Disease rosette formation by Reed-Sternberg cells was observed with the EA reagent. The peripheral blood lymphocytes of 4 patients with chronic leukemia and erythroderma bound the E reagent and are of T lymphocyte origin. In one patient the malignant cells also bound EAC and therefore these cells have receptors of both B and T cells. The application of these techniques to the classification of malignant lymphoreticular cells may lead to important theoretic and therapeutic advances.

147. AN EVALUATION OF MALIGNANT CELL ORIGIN IN LYMPHOSARCOMA OF CHILDHOOD. Castleberry, R. F. *, Zobe, R. K. *, Moreno, H. Children's Hospital, University of Alabama Medical Center, Birmingham, Alabama. Two children with lymphosarcoma (LSA) diagnosed by biopsy of a primary tumor have recently been studied in an attempt to conclusively define the neoplastic cell as thymic (T) or bursal-equivalent (B) in origin. The course of both patients was complicated by meningeal leukemia which, by sampling cerebrospinal fluid, provided an abundant source of leukemic cells. After collecting samples, washing, and adjusting the final cell concentration to 7.5 X 10^6 cells/ml, the following studies and results were obtained: 1) By morphologic criteria on Wright's stain, 100% of cells were lymphoblasts with 95% viability by Trypan blue exclusion. 2) No rosettes were formed when cells were incubated with sheep erythrocytes in a cold environment. (E. rosette assay for T lymphocytes). 3) Using an Fc receptor technique for identifying B lymphocytes, less than 1% of cells were found to have fluorescent surface aggregated IgG. 4) With a complement mediated cytotoxicity assay, more than 95% of the lymphoblasts were lysed with two antisera against human T lymphocytes (one antiserum was prepared against peripheral T lymphocytes from a boy with X-linked agammaglobulinemia and the other to fetal thymocytes). In contrast to adult LSA in which the cells have been reported to have B determinants, this data suggests that LSA cells in children lack B lymphocyte markers but express antigens reactive with antibodies to normal peripheral T lymphocytes. Even though this represents T cell characteristics, because of the cytotoxic activity found with fetal thymocyte antiserum, the possibility that LSA in children is a stem cell disease cannot be excluded.
DIFFERENTIAL EFFECTS OF IRRADIATION ON CULTURED T AND B LYMPHOID CELLS. Han, T., Pauly, J.* and Minowada, J.* Roswell Park Memorial Institute, Buffalo, New York.

It is generally known that lymphocytes are the most radiosensitive cells of the body. However, it has recently been reported that the immunoglobulin-producing human cultured lymphoid cells (B cells), contrary to general belief, are not extremely radiosensitive, as assayed by the colony-forming method. The present report describes a comparative study of irradiation effect on growth pattern of human cultured T and B lymphoid cells. In each experiment, both T and B lymphoid cells were treated with various doses of irradiation (100 - 4,000 r) and the growth patterns of these cells were compared by the 3H-thymidine incorporation method and the viability test at various days (1-4) of incubation after irradiation. Lethal effect of irradiation was expressed as per cent inhibition or per cent reduction, which was calculated by comparing the counts per minute or viable cell counts in irradiated and unirradiated cultures, respectively. A higher per cent inhibition was observed in each and every T cell culture as compared to that of B cell culture expressed as per cent inhibition or per cent reduction, which was calculated by comparing the cant per cent reduction of viable T cells (95 cells, counts per minute or viable cell counts in irradiated and unirradiated cultures, respectively. The most significant difference of per cent inhibition between T and B cells (p<0.05 - <0.01) was noted at each irradiation dose level on day 3 of incubation. A significant per cent reduction of viable T cells (95 - 100%) was observed at 250 r level, whereas irradiation at this dose level killed only 2 - 7% of B cells at day 1-4 of incubation. Our data clearly indicate that cultured T lymphoid cells are extremely radiosensitive and B lymphoid cells, on the other hand, are fairly radioresistant.

THYMIDINE REUTILIZATION IN LYMPHOPOIETIC TISSUES OF NEWBORN MICE. Joel, D.D., Chanana, A.D., Cronkite, E.P. and Malik, D.D.* Medical Dept., Brookhaven National Laboratory, Upton, N.Y. Cytokinetic and intrathymic DNA labeling studies in newborn mice indicate a massive migration of cells from the thymus to peripheral lymphoid tissues. Whether there is significant cell death within the thymus during the neonatal period remains controversial. If cell death occurs in the thymus and other lymphopoietic organs of newborn mice, reutilization of thymidine is possible. By comparing the renewal rate of DNA labeled with 3HThdR to that of 125IUDR, a poorly reutilized thymidine analog, the degree of reutilization can be estimated. Newborn mice were injected subcutaneously with either 125IUDR (1 μCi/gm) or 3HTdR (0.25 μCi/gm). Groups of 20 mice were killed on days 1, 2, 3, 5 and 8 after injection. Total radioactivity of formalin fixed thymus, mesenteric lymph node, spleen, bone marrow and kidney was measured. In the thymus the activity of 125I and 3H remained relatively constant for about 2 days and then declined exponentially. However, the halving time for 3H was 8-10 days compared to 1.5 days for 125I. In the spleen and bone marrow the curves diverged sooner. Lymph node 3H activity steadily increased while 125I activity declined following a temporary increase on day 2. These results strongly suggest significant cell death and thymidine reutilization in the thymus, spleen and bone marrow of newborn mice. The findings support migration of cells from the thymus to peripheral lymph nodes but also indicate that cell death with reutilization of TdR occurs. Parallel decreases of 3H and 125I in the proliferating kidney suggests that there is minimal systemic reutilization.

DEFECTIVE LYMPHOCYTE RESPONSE TO PHYTOHEMAGGLUTININ IN UNTREATED HODGKIN'S DISEASE. Ziegler, J.B.* Hansen, P.*, Penny, B.*, (Intr. by L.A. Boxer). University of New South Wales and St. Vincent’s Hospital, Sydney, New South Wales, Australia. Defective cell-mediated immunity is a well recognized feature of Hodgkin’s disease; most data, however, has been obtained from previously treated patients. A study was conducted in 27 newly diagnosed untreated patients at presentation: delayed hypersensitivity was assessed by the intradermal injection of candida, mumps, streptokinase/streptodornase, and PPD antigens; peripheral blood lymphocytes were cultured in the presence of a range of phytohemagglutinin (PHA) concentrations from 0 to 800 μg/ml. Fourteen patients were in stages 1, 2, or 3A and of these, 4 (36%) were anergic. Of 13 patients, in stages 3B or 4, 8 (62%) were anergic. Delayed hypersensitivity responses did not distinguish patients in these two staging groups (P<0.1). Peripheral lymphocyte counts were normal in all stage 1 - 3A patients and reduced in 10 of 13 (77%) stage 3B - 4 patients (P<0.01). PHA responses were reduced in 4 of 14 (29%) patients in stages 1 - 3A and in 12 of 13 (92%) patients in stages 3B - 4 (P<0.005); serum factors did not account for observed abnormalities in PHA response. All of the 10 lymphopenic patients showed a reduced lymphocyte response to PHA and in 7, the response at all PHA concentrations employed. However, in 2 patients, including 5 with normal lymphocyte counts, the lymphocyte response was reduced only at low PHA concentrations and was normal at high concentrations. This study shows the lymphocyte dose-response to PHA to be a sensitive index of a lymphocyte defect in Hodgkin’s disease and is a useful adjunct in clinical staging. The results indicate that a functional T lymphocyte defect is a frequent finding in untreated patients and appears to precede the development of lymphopenia.

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151.

POLY(A) POLYMERASE IN NORMAL AND PHA STIMULATED HUMAN LYMPHOCYTES. Coleman, M.S.* and Hutton, J.J. University of Kentucky Medical Center. Lexington, Kentucky.

Poly riboadenylate (A) polymerases have been reported to catalyze the incorporation of multiple AMP residues into a certain fraction of total heterogeneous nuclear RNA. These sequences are conserved in processing heterogenous nuclear RNA to messenger RNA and may be important in distinguishing messenger RNA's from other classes of RNA. We have examined normal and phytohemagglutinin (PHA) stimulated human lymphocytes for enzymes which incorporate terminal AMP residues onto the 3'-OH group of an oligo(A) initiator and have no requirement for a template chain. The extent to which RNA processing is limited to the nucleus is not known, but our poly(A) polymerase activity is located almost exclusively in the cytoplasm rather than in the nucleus. This specific cytoplasmic poly(A) polymerase is called terminal riboadenylate transferase (TrT) to differentiate it from a reported nuclear poly(A) polymerase. It is soluble, Mn^2+ dependent, and has a MW of approximately 65,000 by velocity sedimentation. A similar activity has been purified from calf thymus Tsiapalis et al. BBRC 50:737 (1973). During PHA activation of human lymphocytes TrT activity increases 5-fold (from 18 in unstimulated cells to 90 moles rATP incorporated/10^6 cells/hr in stimulated cells). The activity peak occurs at 45 hours after introduction of PHA and coincides with maximal blast conversion. Induction of activity is completely inhibited by introduction of cycloheximide at zero time (70% inhibition in protein synthesis after 44 hrs.). In vivo, TrT differs from DNA dependent RNA polymerase by its resistance to inhibition by rifampicin and cycloheximide. TrT appears to be distinct from nuclear poly(A) polymerases and may play a role in the proliferation and differentiation of lymphocytes.

152.

RNA IN NASCENT DNA FROM PHYTOHEMAGGLUTININ-STIMULATED HUMAN LYMPHOCYTES. Mendelsohn, J., Fox, R.M.*, VandeVenter, L.*, Goulian, M. University of California, San Diego, School of Medicine, La Jolla, California.

Studies in a variety of organisms indicate that DNA is synthesized in short nascent strands which are rapidly incorporated into the growing molecule. However, until recently, the mechanism for initiation of DNA replication has been poorly understood. Studies with microorganisms and two recent studies of permanent cell lines indicate that short RNA strands act as primers for DNA synthesis in vivo, and are apparently linked covalently to nascent DNA. In the present study, DNA synthesis was studied in short term cultures of normal human peripheral lymphocytes. Sixty hours after adding phytohemagglutinin, the lymphocytes were incubated with short pulses of 3H-thymidine at 27°C. Nascent DNA purified from lysed cells behaved as single-stranded DNA on hydroxylapatite chromatography. The single-stranded species had a mean size of 152. RNA is associated with nascent DNA through a covalent linkage, is demonstrated a mean density slightly greater than reference DNA. After treatment with RNase or alkali, the density shifted to that of the reference marker, suggesting that the increased density was due to RNA associated with the nascent DNA. This conclusion was confirmed by similar studies of nascent single-stranded DNA isolated by a different procedure, using nitrocellulose column, and by showing incorporation of 3H-uridine into this nascent DNA. It is concluded that in unstimulated human peripheral lymphocytes, as in permanent cell lines and microorganisms, RNA is associated with nascent DNA through a covalent linkage, consistent with function of the RNA as primer for synthesis of DNA.

153.


The cytokinetic characteristics of human peripheral blood lymphocytes in phytohemagglutinin stimulated cultures were examined by means of new autoradiographic techniques which assay the fraction of cells whose nuclei contain DNA polymerase, utilizing endogenous (PDP), or exogenous (DDDP) DNA primer-template capability. The fraction of cells measured by the PDP assay is equivalent to the Growth Fraction (GF) in systems, like the cultured lymphocytes, with a short cell cycle and a short G1 period. Both PDP and DDDP activity increases in stimulated lymphocytes 5 to 11 hours prior to the start of DNA synthesis. Average estimates of the GF, cell cycle time, and G1 period, respectively, for the first 4 days in culture are as follows: Day 1 - 0.10, 66.7 hrs, 53.7 hrs; Day 2 - 0.30, 14.0 hr, 1.0 hr; Day 3 - 0.485, 14.9 hr, 1.9 hr; Day 4 - 0.515, 15.8 hr, 2.8 hr. By use of the DDDP technique, evidence is presented to support the hypothesis that daughter cells of the first divisions may be retired from cycle by virtue of loss of primer-template availability, rather than by loss of DNA polymerase.
AN EVALUATION OF LEUKEMIC SERUM INHIBITORS OF PHA. Humphrey, G.B., Lankford, J.*, Croome, A.*

The influence of leukemic serum on phytohemagglutinin (PHA)-induced lymphocyte transformation was studied. All sera were isolated from children with acute lymphoblastic leukemia (ALL). Twenty-two of 24 pretreatment sera collected at diagnosis inhibited the transformation of normal lymphocytes stimulated with PHA, the mean response being only 10% of normal. In these experiments with PHA, kinetic studies of transformation demonstrated that leukemic serum inhibited rather than delayed transformation. The inhibition of transformation by PHA was assumed to be due to the binding of PHA by components of leukemic serum, as the inhibition could be overcome by an increase in the PHA concentration. Furthermore, semi-quantitative experiments which assessed the binding of PHA to lymphocytes further substantiated this mechanism. Double diffusion studies of PHA versus leukemic serum or normal serum in Ouchterlony plates and by immunoelectrophoresis suggested that more than one component of leukemic serum may bind PHA and the differences between leukemic serum and normal serum may be both quantitative and qualitative in nature. That the presence of these inhibitors in leukemic sera may be of prognostic significance has been suggested by the observation that the inhibitory capacity of leukemic sera collected during clinical remission was consistently less than that of the respective diagnostic sera in 11 of 12 patients. A quantitative assay of inhibition has shown, in one case, that the concentration of inhibitors decreased after diagnosis, remained low until just prior to relapse and was elevated at the time of relapse. Further studies of leukemic serum inhibitors are necessary to determine whether these observations will be of prognostic significance in following patients with ALL. Such studies are currently in progress as well as studies designed to examine the relationship of the inhibitors to alpha-2-macroglobulins.