Immunologically Specific Antigens in Leukemic Tissues

By Irving Greenspan, Eric R. Brown and Steven O. Schwartz

Previous studies have demonstrated that rabbits develop antibodies against both human and mouse leukemic tissues. Antibody reactions were also demonstrated in man following the injection of extracts of human leukemic brain. The present studies were undertaken to confirm previous results, to broaden the experiments, and to develop technics of in vitro testing for antibodies which are specific to the leukemic tissues, and more specifically to the leukemogenic agents. This report encompasses the results of these investigations. They confirm that a specific immunologic response can be elicited in both man and rabbit by leukemic human tissues. The technics used to demonstrate the presence of antibodies included passive cutaneous anaphylaxis (PCA), immunodiffusion, microprecipitin, and immunofluorescent tests.

Materials and Methods

Preparation of Human Antisera (Antibodies)

Antibodies in subjects inoculated with cell-free extracts of leukemic human brains had been demonstrated in the past by the technic of passive cutaneous anaphylaxis. In the present study volunteers were divided into three groups: one group received human leukemic cell-free brain extract; one group a cell-free extract made from a Hodgkin's disease node, and the third group, human non-leukemic cell-free brain extract. All volunteers were examined before, during and after the immunization series. Particular attention was paid to the blood and the nervous system in view of the nature of the inoculum. None of the examinations revealed significant changes, and no late sequellae have developed over a 4-year observation period.

The preparation of antibodies in man was carried out as follows:

1. Five per cent pooled homogenates of frozen leukemic human brain, normal human brain or a single Hodgkin's disease node were prepared in buffered physiologic saline solution by grinding the tissues in a mortar with sand. The resulting homogenates were passed through a No. 6 Seltz sterilizing filter. The filtrates were individually stored at -20 C. until used.

2. Blood samples were drawn from all volunteers for control studies and at the time of each inoculation.

3. Nine men were given leukemic brain filtrate, two Hodgkin's disease node filtrate, and three non-leukemic brain filtrate. The dose was 0.1 cc. intracutaneously and 0.9 cc. intramuscularly.

4. Three and 6 weeks later the inoculations were repeated.

5. One month later each man received an intramuscular injection of one ml. adjuvant.
Table 1.—Summary of PCA, Microprecipitin, Immunodiffusion Tests

<table>
<thead>
<tr>
<th>Serum</th>
<th>Antigens</th>
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<tbody>
<tr>
<td></td>
<td>NHB</td>
</tr>
<tr>
<td>I. Volunteers Immunized against:</td>
<td></td>
</tr>
<tr>
<td>1. Normal human brain</td>
<td>+</td>
</tr>
<tr>
<td>2. Leukemic human brain</td>
<td>± ±</td>
</tr>
<tr>
<td>3. Hodgkin’s disease lymph node</td>
<td>−</td>
</tr>
<tr>
<td>4. Normal human brain*</td>
<td>−</td>
</tr>
<tr>
<td>5. Normal human brain (absorbed with leukemic R.E. antigen)</td>
<td>±</td>
</tr>
<tr>
<td>6. Leukemic human brain*</td>
<td>−</td>
</tr>
<tr>
<td>7. Leukemic human brain (absorbed with leukemic brain)</td>
<td>−</td>
</tr>
<tr>
<td>II. Exposed Personnel:</td>
<td></td>
</tr>
<tr>
<td>1. Before exposure to leukemia</td>
<td>−</td>
</tr>
<tr>
<td>2. After 6 months or more exposure</td>
<td>−</td>
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<tr>
<td>III. Leukemic Patients</td>
<td></td>
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<tr>
<td>IV. Rabbits Immunized against:</td>
<td></td>
</tr>
<tr>
<td>1. Pre-immunized</td>
<td>−</td>
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<tr>
<td>2. Normal human brain</td>
<td>++</td>
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<tr>
<td>3. Normal human brain*</td>
<td>−</td>
</tr>
<tr>
<td>4. Leukemic human brain</td>
<td>± ±</td>
</tr>
<tr>
<td>5. Leukemic human brain*</td>
<td>−</td>
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</tbody>
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NHB = normal human brain; LHB = leukemic human brain; HLRE = human leukemic reticuloendothelial tissues; F = Freon-extracted leukemic human antigen; HDN = Hodgkin’s disease node; NMB = normal mouse brain; LMB = leukemic mouse brain.

*Antisera absorbed with normal human brain.

Weakly positive.

(1.3 ml. of filtrate and 4 ml. of Arcel*) combined with antigen. The final bleeding took place 3 weeks later.

Other sera were studied from normal persons, laboratory personnel exposed to human and animal leukemia, and from persons convalescing from various virus diseases (influenza, herpes simplex, hepatitis, poliomyelitis).

Absorbed sera: Both the normal human brain and leukemic brain antisera were absorbed with normal human brain or liver by the method of Coons before testing. Natural isoantibodies and isoantibodies to tissue antigen components were looked for in the sera before immunization by cross adsorption studies utilizing PCA, microprecipitin and immunodiffusion tests. The reactions of the sera from the volunteers who were inoculated with normal tissues were compared to those who received leukemic antigens; and cross adsorption studies were also done using the human leukemic antibody and normal tissue antigens (see table 1).

Preparation of Antigens

1. Human brain antigens: Ten per cent homogenates of normal or leukemic human brains were aseptically prepared with sterile saline solution in a blender. The preparations were passed through a Seitz sterilizing filter and either frozen or lyophilized for future use.

2. Fluorocarbon extracted antigens: Freon 113* extractions of normal and leukemic human tissues were prepared by the method of Stone and Moore.5

3. Normal human lymph node and Hodgkin's disease lymph node antigens: These tissues were prepared as 10 per cent homogenates similarly to the brain antigens.

**Testing of Antigens and Antisera**

1. Passive cutaneous anaphylaxis: One ml. of lyophilized serum was reconstituted with saline solution and injected intraperitoneally into albino guinea pigs (approximately 250 Gm.). One-tenth cc. of the antigen was injected intracutaneously 48 hours later. Equally satisfactory results were obtained when the antigen was injected intraperitoneally and 0.1 ml. of the antibody was injected intracutaneously 17 hours later. In the performance of the test, 0.6 ml. of 0.5 per cent Evans blue dye is injected either into the hind vein of the foot

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*Freon 113 was obtained through the courtesy of Dr. F. S. Blodgett, E. I. DuPont de Nemours & Co., Wilmington, Del.
Fig. 2.—Immunodiffusion test. (A) Ascites fluid (antigen); (1) serum, rabbit: anti-mouse ascitic fluid; (2) serum, human: anti-leukemic human brain; (3) serum, human: anti-non-leukemic human brain; (4) serum, rabbit: normal; (5) serum, human: normal. (Note: Only common reactions with sera 1 and 2.)

by the method of Kabat and Mayer, or by the intracardiac route simultaneously with the skin injection.

The optimal ratio of antigen to antibody had to be determined by trial and error. Excess of either antigen or antibody results in cross reactions with normal tissue antigen, leading to false positive reactions due to organ specific antibodies.

2. Immunodiffusion: For the Ouchterlony gel diffusion reaction, the medium was one per cent Difco-Nobel agar in phosphate buffered physiologic saline solution, with 1 per cent glycerin added after the initial cooling. Before pouring of plates, merthiolate 1:10,000 was added. A seven-hole standard Feinberg agar gel cutter was used, and the holes were underlaid with diffusion agar before the addition of antigen or antibody. Antigen, 0.4 ml., was placed in the central hole, and 0.3 ml. of the sera to be tested were placed in the six surrounding holes (fig. 2). Serial dilutions of test antigens or sera could be examined by this technic. A critical ratio must exist between antigen and antibody before a reaction manifests itself. This ratio was determined by using positive sera against positive antigens, using dry weights for standards.

3. Microprecipitin tests: Alkali-free noncorrosive glass (70 mm. by 0.5-0.9 mm. internal diameter) capillary tubes were used in a manner similar to those used for typing streptococcal antigens. Soluble antigens were concentrated to a McFarland No. 10 standard. The test serum was drawn up to the midpoint of the capillary tube, the outside wiped to prevent serum from entering the antigen container, and the antigen drawn into the tube. The full tube was inverted and inserted into a clay-bank holder and allowed to stand for 30 minutes. A solid white precipitate at the midpoint was read as a positive reaction. The presence of particulate matter along the length of the tubing was considered doubtful. The doubtful readings were re-tested for final evaluation. The test has high specificity but low sensitivity, and for that reason titers could not be determined with accuracy. The test has limited usefulness as a fast screening method.

4. Immunofluorescence: A Reichert Fluorex microscope with mercury arc lamp was
used in these studies. Sera were adsorbed by the method of Coons,\textsuperscript{4} tested for specificity, and conjugated with fluorescein isothiocyanate dye. The method of conjugation was that described by Riggs,\textsuperscript{7,8} as modified by Brown and Bittner\textsuperscript{9} and the Lissamine-Rhodamine RB-200 counterstain technic of Smith and his co-workers.\textsuperscript{10} Laboratory purified dyes are more specific and less likely to show nonspecific fluorescence than those prepared by commercial dye manufacturers because of the refinements in technics allowed by small scale production.\textsuperscript{10,11} Chadwick, McEntegart and Nairn\textsuperscript{12,12a} as well as Hughes\textsuperscript{13} have shown that the use of Lissamine-Rhodamine RB-200 counterstains would eliminate nonspecific fluorescence in tumor and normal tissues when used with fluorescein isocyanate. Smith and co-workers\textsuperscript{10} have shown the same for isothiocyanate specific conjugated dyes. This proved highly successful in negating the natural fluorescence of tissues in these studies also. Normal and leukemic human and mouse spleen, liver, kidney and brains were examined. Tissues were prepared by either direct impression or by the method of Smith, et al.\textsuperscript{10}

\textbf{RESULTS}

A positive reaction could be demonstrated by passive cutaneous anaphylaxis when the serum from an immunized subject was injected intraperitoneally into the guinea pig, and the challenging antigen was obtained from either human or mouse leukemic tissue. Positive results were also obtained when leukemic tissue antigens were used to sensitize, and immunized human antisera were used to challenge. All other combinations of sera and antigens gave negative results.

The specificity of the reaction was retained after absorption with normal human or mouse brain but was lost by the absorption with leukemic human or mouse brain. Extracted gamma globulin from positive sera gave a strong positive reaction.

The sera of the nine men injected with leukemic brain extract reacted positively with leukemic human brain, leukemic mouse brain, and fluorocarbon-extracted leukemic brain. Sera from the same men collected before immunization failed to react with the same antigens. Both the positive and negative reactions were consistent when the proper proportions of antigen and antibody were used. Under these conditions neither false positive nor false negative reactions were encountered.

The anti-leukemic sera gave negative results with extracts of carcinoma tissues and a host of viral agents such as herpes simplex virus, influenza virus and poliomyelitis virus.

Serum obtained from the two men injected with Hodgkin’s disease node extract gave positive reactions with Hodgkin’s disease node extract, and a less intense reaction with leukemic tissue extracts. They had given negative reactions prior to inoculation. Reactions failed to appear with any of the other antigens.

Further evidence of specificity was demonstrated in six persons working with the leukemic mice and leukemic human tissues. Their sera initially failed to react with either leukemic brain extract or normal brain extract. After 6 months of working with experimental leukemia, their reactions became positive to the leukemic extracts, though in general their titer remained lower than the injected group’s.

Sera obtained from 24 normal adults and six children were negative to both leukemic and normal brain extracts.
Sera obtained from six adults and four children with acute leukemia gave repeatedly negative results with all antigens.

The three men injected with non-leukemic human brain extract reacted strongly positive with non-leukemic human brain extract, and weakly positive with leukemic human brain extract, indicating organ specificity. This reaction became negative to both non-leukemic and leukemic brain extracts when the serum was absorbed with non-leukemic brain extract. The non-adsorbed serum from these individuals did not react positively with Hodgkin’s disease node extracts.

Immunodiffusion Test

The immunodiffusion test was carried out on the following three groups of test sera: (1) the volunteers, (2) exposed laboratory personnel, and (3) sera from rabbits immunized with normal and leukemic human tissues. The method described by DeCarvalho14 was used to determine reactivity.

As in the PCA test, optimal ratios of antigen to antibody were necessary, and were essentially obtained by trial and error. In this system minor bands of antibody, common to normal tissues, were detected along with the stronger bands to the leukemic antigens. The number of precipitin bands to appear on a plate varied with the system under study. When normal tissue antibodies were compared to leukemic tissue antibodies at high concentrations, common bands were noted between them. In these cases, however, the leukemic antisera had at least one distinctly different band.

When the test is carried out in the manner described, it is both a highly specific and highly sensitive test for the demonstration of antibodies which are specific in both human and rabbit sera (table 1).

Microprecipitin Tests

Results of the microprecipitin tests can be divided into three groups: the volunteers, exposed personnel and rabbit sera. Consistent results were obtained when the antisera concentration was optimal when tested against its antigen. Under these conditions rapid distinction could be made between the leukemic and normal series. The human anti-leukemic and human anti-Hodgkin’s disease node sera reacted positively against human leukemic brain, human leukemic spleen and fluorocarbon-extracted leukemic antigen, but failed to react with normal human tissue antigens.

In general the results agree with those for the immunodiffusion method. The microprecipitin test has been valuable in screening for antibody-antigen reactions. It has the advantages of simplicity and rapidity, but has the disadvantage of lack of sensitivity.

Immunofluorescence

The results of the immunofluorescence test showed that fluorescein isothiocyanate conjugated human anti-human leukemic sera (pooled from volunteers) reacted, when unadsorbed, against both normal and leukemic human brain impression smears. A greater degree of fluorescence, 4+ as compared
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to 1 to 2+, was found when these conjugates were tested on leukemic brain. When anti-leukemic sera were conjugated after adsorption with normal human liver or brain, fluorescence was only demonstrable in leukemic tissues. The fluorescence appeared in the cytoplasm adjacent to the nuclear membrane and resembled inclusion bodies. When adsorbed gamma globulin was fractionated from anti-leukemic sera, fluorescence was also seen only with leukemic brain impression smears. The adsorbed human anti-leukemic sera showed a 3+ reaction when tested against leukemic mouse brain impression smears. They failed to react with any normal mouse tissues. The highest degree of fluorescence was always elicited by a homologous system.

DISCUSSION

A review of the literature on the passive cutaneous anaphylaxis test shows that when the skin is sensitized with cell-fixed antibodies, the injection of an antigen elicits an immediate reaction of the urticarial type. Chase observed erythema and wheal formation following the injection of antigen and antibody, but Ovary found that these reactions could be more readily demonstrated by injecting a dye simultaneously with the antigen. When this is done, the dye accumulates at the site of the reaction, probably as a result of the release of histamine and the increased permeability of minute vessels which allow the exudation of the dye.

Passive cutaneous anaphylaxis was demonstrated when either immunized human sera or immunized rabbit sera were used to sensitize, and the antigen used to challenge was obtained from leukemic human tissues. All other combinations of antigens or sera gave negative results. Those persons whose sera were tested before and after immunization had a negative reaction before immunization and a positive reaction afterward. To control the experiments, antigens and dye were injected without antiserum, and immune sera and dye were tested with saline solution as the antigen: these experiments all yielded negative results. Different sites were tried on the animal’s back, and the order of injections was varied to eliminate the possibility of certain positions or order of injections favoring positive results.

It is recognized that not all human sera can sensitize a guinea pig’s skin. This is because either the antibody titer is too low, or the antibody is not of a skin-sensitizing type. Some of the negative results might be due to either of these reasons; however, the results with the other tests would negate these objections.

Passive cutaneous anaphylaxis (fig. 1) is an extremely sensitive test for antibody. When using serum from human beings injected with leukemic brain extract and high levels of non-leukemic antigen, positive reactions were obtained. When the amount of non-leukemic antigen was reduced, these same sera no longer gave positive reactions. Positive reactions were obtained, however, when these sera were tested against leukemic antigens at the same dose level. Thus, organ specificity could be ruled out as responsible for the reactions. At least two separate antigens are evidently present in the leukemic brain extract. (Note: By the Ouchterlony method at least three bands were seen.)
This was also demonstrated by the ability of leukemic human brain filtrate to adsorb the activity of the reaction of previously positive sera. Normal human brain filtrates will not negate the activity of positive sera.

Under optimal conditions, the immunodiffusion technic offered the highest degree of specificity. In this system minor bands of antibody common to normal tissues were detected along with the stronger bands to the leukemic antigens. The leukemic band is the only one that blends with both human and mouse antisera (fig. 2). Berenbaum and co-workers\textsuperscript{19} have pointed out that non-specific reactions may occur when the immunodiffusion tests are carried out at low temperatures. At 37 C, nonspecific reactions did not occur in our studies.

The microprecipitin test has the advantages of being highly specific and relatively simple to perform. Its lack of sensitivity, however, largely negates these advantages. However, the specificity of the reaction is such that either the antibody or antigen may be demonstrated by this test. For this reason the sera were initially screened by this method. No false positive reactions were found with normal tissues.

Where immunofluorescence studies were done, they confirmed the results of other methods. These tests served to emphasize the degree of cross-reactivity between leukemic human and leukemic mouse tissues. They also demonstrated the difference between normal and leukemic tissues of both human and murine origin. The fact that the entire leukemic cell did not uniformly fluoresce would indicate that the antileukemic antibody reacted not against the cell, but against a component within the cell which is not present in normal cells.

The successful concentration and purification of viruses from tissues by means of fluorocarbon extractions has been reported by numerous workers. Stone and Moore\textsuperscript{5} reported the purification of the mouse mammary carcinoma agent by means of fluorocarbon such as Freon 113 and Genetron 226. Taylor\textsuperscript{20} and DeCarvalho\textsuperscript{14} have reported that antigens could be obtained from fresh tumor tissues and tumor cell lines of human and chick origin by treatment of extracts with fluorocarbon.

The recent article by McKenna\textsuperscript{21} and associates is of interest. They obtained antigens from HeLa and HeLa J 111 cells by treatment with fluorocarbon. Absorption studies indicated that the antigen from HeLa cells had no serologic relation to normal human uterus, but “antibody for HeLa extract coated red blood cells was found in 25 per cent of the sera from patients with malignant disease.” Both Taylor and DeCarvalho reported that antigens obtained by fluorocarbon extraction were specific for the original tumor when assayed by complement-fixation and immunodiffusion tests against immune rabbit sera. The work with fluorocarbon extractions suggested a means by which an interference mechanism might be bypassed in order to isolate the virus capable of producing leukemia.

Rabbit sera prepared against extracts made from leukemic human and mouse tissues indicate that a close antigenic relationship exists between them. Garb, Stein and Simms,\textsuperscript{22} using immunologically tolerant rabbits, have produced antibodies that react with human leukemic antigens. In these studies,
newborn animals were injected with normal human blood, producing immunologically tolerant animals. The subsequent immunization of these animals with human leukemic blood produced antibodies only to the leukemic component of the blood. The specificity of the reactions which they describe, as demonstrated by immunodiffusion technics, correlates well with those reported in this paper. Using technics similar to those of Garb, Stein and Simms, we have produced antibodies in immune tolerant animals following the inoculation of leukemic and Hodgkin's disease tissues. The fact that the resulting antibodies failed to react with comparable normal tissues indicates that organ specificity is not responsible for the reactions described.24

The specificity of the reaction described in these experiments adds confirmation to the thesis previously advanced24 that antibodies are produced against filtrates and extracts of leukemic tissues. There is ample evidence that there is a difference between the antigenic behavior of normal and leukemic tissues. The most obvious explanation of this is that the leukemic cells, by virtue of their enzymatic, chemical or other alterations, are capable of eliciting a different response from the normal. Had leukemic tissues or tissues containing leukemic cells been used throughout these experiments, this explanation would suffice. There would be no problem of reconciling the various types of responses in either man or rabbit, the explanation of the results of adsorption or cross adsorption experiments, or the apparent high degree of specificity demonstrated.

This explanation, however, does not take into account some of the observations, especially those made on individuals exposed to either leukemic patients or leukemic mice. Why should physicians continuously in contact with leukemic patients, mothers caring for their leukemic children,24 and laboratory workers in contact with leukemic animals develop an antibody response, lower in titer, but indistinguishable from that obtained in men inoculated with extracts of leukemic tissues or rabbits inoculated with either leukemic tissues or leukemic tissue extracts? A logical explanation is that the agent—the virus—responsible for the production of the leukemia in both man and the mouse is itself sufficiently antigenic to account for this antibody production.

This explanation immediately poses new questions of its own. If the virus is so readily transmitted, why do not the new hosts, both those purposefully inoculated and those environmentally exposed, develop leukemia? Why do leukemic patients lack antibodies? What is the relationship between leukemia and Hodgkin's disease in view of the similarities in response elicited by the materials obtained as antigens from these two conditions?

It is obvious that at the present time, with our factual knowledge as yet so incomplete, many of these questions can only be answered theoretically and by drawing on analogies from other established and more completely understood systems of virus diseases. That leukemia is difficult to transmit even by the use of cells, except into genetically histocompatible hosts, is well established. The ready immunization of mice would be a further barrier. The genetic differences in man and his ability to form antibodies as shown in the foregoing experiments are ample reasons in themselves for the failure of the
new hosts to develop leukemia. Furthermore, it is likely that the leukemia virus is fairly ubiquitous and minor antibody responses occur quite early without the demonstrability of antibodies. In studying families of patients with leukemia for antibodies, it has been found that not all members of a family have antibodies demonstrable by the technics at present available. This, of course, does not necessarily mean that the antibodies are not present at concentrations below demonstrability and that further antigenic challenge will not result in adequate protective antibody levels appearing.

Why patients with leukemia lack antibodies may have at least two explanations. It may be that the leukemia functionally incapacitates or overpowers that portion of the lymphoid or reticuloendothelial system responsible for the production of adequate quantities of protective antibodies. Or, a view we find more attractive, individuals who develop leukemia are incapable, by virtue of genetic or other reasons, to respond adequately to the challenge of the leukemogenic virus.

That there should be such a striking similarity between the antigenic behavior of Hodgkin's disease tissues and leukemic tissues is hardly surprising if one is willing to accept the postulate that the morphologic manifestations merely represent the response of the host to the challenge.

Albeit these interpretations are theoretical and as yet not capable of proof, there is no evidence to refute them and they serve as a basis for explaining the facts accumulated. Their substantiation must await the isolation and propagation of the virus in a form free from the contaminating proteins of their original tissues.

There has been some question relative to the wisdom of injecting volunteers with brain extracts in view of the possibility of producing an allergic encephalomyelitis. Evidence indicates that unless Freund's adjuvant-combined or alcohol-extracted brain or central nervous tissue is employed, encephalomyelitis is not likely to occur. Raffel has summed up this question as follows; "Experimental encephalomyelitis may be a disease predicated upon delayed allergic reactivity to a protein of nerve tissue. . . . This is a highly speculative sequence, not clearly supported by sufficient understanding of experimental facts. . . ."

**Summary**

The injection of extracts of leukemic and Hodgkin's disease tissues of man elicits an antibody reaction in both man and rabbit. This response is similar to that elicited by leukemic mouse tissues when injected into rabbits. The antibody reaction may be demonstrated by the technics of passive cutaneous anaphylaxis, immunodiffusion, microprecipitin and immunofluorescence. Tissue extracts from non-leukemic individuals do not elicit a similar response. Rabbits immune-tolerant to normal human tissues produce antibodies specific to leukemic human antigens. Antibodies develop in those individuals who are exposed for a long time to either human or mouse leukemia.

These immunologic studies demonstrate specific antigenic differences between normal and leukemic tissue extracts. It is postulated that the difference
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between normal and leukemic extracts is the consequence of the presence of viruses or the alterations caused by them.

SUMMARIO IN INTERLINGUA

Le injection de extractos ab tissu afficite de leucemia o de morbo de Hodgkin in humanos evoca un reaction de anticorpore tanto in le homine como etiam in conilios. Iste responsa es simile a illo evocate per leucemic tissu murin quando injicite in conilios. Le reaction anticorporee pote esser demonstrate per medio del technicas de passive anaphylaxia cutanea, immunodiffusion, microprecipitina, e immunofluorescentia. Extractos de tissu ab non-leucemic subjectos non evoca un simile responsa. Conilios que es immuno-tolerante pro normal tissu human produce anticorpore specific pro leucemic antigeno human. Anticorpore se disveloppa in ille individuos qui es exponite prolongatemente a leucemia human o murin.

Iste studios immunologic demonstra specilc differentias antigenic inter extractos de tissu normal e leucemic. Es postulate que le differencia inter normal e leucemic extractos es le consequentia del presentia de virus o del alterationes causate per virus.

ACKNOWLEDGMENTS

The authors wish to acknowledge their indebtedness to Warden Jack Johnson, former Sheriff Joseph P. Lohman and Sheriff Frank F. Sam for their cooperation in the procurement of the volunteers. The assistance of Mr. LeRoy Yates, Mr. Seymour Greenspan and Dr. Allen Feinberg in various phases of the work is also gratefully acknowledged.

REFERENCES


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