Abnormal Nucleic Acid Metabolism of Lymphocytes in Plasma Cell Myeloma and Macroglobulinemia

By Sydney E. Salmon and H. Hugh Fudenberg

INCREASED NUMBERS of plasmacytoid cells, associated with the formation of large amounts of monoclonal proteins is characteristic of plasma cell myeloma and macroglobulinemia of Waldenström. Although aberrations of the immune response1 are present in both diseases, patients with myeloma are particularly prone to bacterial infections.1,2 In the malignant lymphoid disorders of chronic lymphocytic leukemia3 and generalized Hodgkin’s disease,4 the transformation response of lymphocytes to phytohemagglutinin (PHA) is impaired. Using the incorporation of radiolabeled nucleosides into lymphocyte deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), we have studied patients with myeloma and macroglobulinemia, and correlated the in vitro cellular responses with serum immunoglobulin levels and past history of infection. The results demonstrate significant impairment of lymphocyte responsiveness to PHA in these disorders.

MATERIALS AND METHODS

Patient Materials

Twenty patients with plasma cell myeloma, and six patients with macroglobulinemia of Waldenström were studied (Table 1). All but one of these patients had monoclonal paraproteins present in the serum or urine, as well as characteristic clinical findings. Five of the myeloma patients were receiving chemotherapy; of the 15 who were not, 10 were studied immediately after the diagnosis was made, and never received chemotherapy, while the others had not received any cytotoxic or hormonal therapy for at least two months before the lymphocyte studies. None of these patients were receiving gammaglobulin injections or sodium fluoride therapy. The macroglobulinemic subjects studied were treated only by intermittent plasmapheresis. All of the subjects studied had serum creatinine values of less than 1.2 mg. per cent; subjects receiving chlorpromazine, diphenylhydantoin, diphenhydramine, or other medications thought to affect lymphocyte function,5 were specifically excluded. Control subjects include 17 normal volunteers in the same age range as the patients, as well as 10 additional patients with diffuse benign hypergammaglobulinemia (greater than 2.0 Gm. per cent γG globulin) of diverse etiologies (acute lupus erythematosus 4, rheumatoid arthritis 2, hypergammaglobulinemic purpura 3, and tuberculosis 1), none of whom were receiving antimetabolite or steroid therapy.

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ABNORMAL NUCLEIC ACID METABOLISM

Culture Preparation

Thirty-five to 50 milliliters of heparinized venous blood was allowed to sediment for two hours, after which the leukocytes in the supernatant plasma were aspirated and washed five times in Spinner salt solution (Grand Island Biological Co., Grand Island, N.Y.) containing 1 per cent bovine serum. The total white blood count of the donors was in the range of 3,000 to 8,000 per cubic millimeter, with differential counts demonstrating 35–58 per cent lymphocytes. Myeloma patients often had mild granulocytopenia. In general, lymphocytes comprised 55–70 per cent of the cells isolated from the plasma supernates. Small numbers of monocytes were also present in the cultures. The total white blood count of the suspensions was 500,000–800,000 per ml. Dextran sedimentation was not employed in these studies. The washed leukocytes were resuspended, enumerated, and cultured at a cell concentration of 1 X 10^6 lymphocytes per 3 milliliters of minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10 per cent bovine serum, glutamine, penicillin and streptomycin.

The cells were incubated at 37°C in 16 X 150 mm. sterile rubber stoppered tubes 15 degrees from the horizontal, gassed with 5 per cent CO2 in air, with regassing after additions (labeled nucleosides, PHA, or metabolic inhibitors). Phytohemagglutinin-M (PHA-M, Difeo Labs, Detroit, Mich.) was the mitogen used in these experiments. In all assays, duplicate tubes were run for each cell source both with and without the addition of PHA. Lymphocyte viability was determined by Trypan blue exclusion and consistently demonstrated 84–97 per cent viability at sixty-eight to seventy hours of culture time. The cells were harvested by centrifugation and successively treated and recentrifuged after additions of 10 per cent trichloroacetic acid, 80 per cent ethanol, and a 1:1 mixture of absolute ethanol and ethyl ether. The acid-insoluble residue was dried, dissolved in 0.5 ml. of 90 per cent formic acid, added to 20 ml. of (a-naphthylphenylazo) ANPO and counted in a Packard Tri-carb liquid scintillation spectrometer (Packard Instruments, Downers Grove, Ill.), utilizing automatic external standardization quench correction technics. The radioactivity data are expressed as counts per minute per 10^6 lymphocytes. Morphologic transformation was not assessed in these experiments. Mean levels of radioactivity are expressed in the text ± the standard error of the mean (S.E.M.) and shown in the figures with the 95 per cent confidence limits for the mean of each group. Comparisons of patient and control groups are based on calculation of composite variance and the standard error of the difference, with a subsequent "T test" applied for significance.

Nucleic Acid Synthesis

The DNA and RNA assay systems used were those previously used to compare patient populations with acquired agammaglobulinemia to normal subjects.6 The DNA assay was performed with the method of Tormey and Mueller.7 Cells were planted with a standard dilution of PHA-M (100 micrograms) and then incubated for 48 hours, at which time 3 X 10^-6 M methotrexate (methotrexate sodium, Lederle Laboratories, Pearl River, N.Y.) was added to inhibit dehydrofolate reductase activity. Adenosine 1.5 X 10^-4M. (Grade A. Calbiochem, Los Angeles, Calif.) was added simultaneously to permit RNA synthesis to continue. Eighteen hours later, the methotrexate block was circumvented by the addition of .044 microcuries of 14C-thymidine (3.66 mc./mM.) (New England Nuclear Corp., Boston, Mass.) to each culture, which was then incubated for an additional six hours incorporation period, before harvesting for scintillation counting.

RNA synthesis was measured by the method of Mueller and Le Mahieu,8 except that 8H-uridine (New England Nuclear Corp., Boston, Mass.) was substituted for 3H-cytidine as the RNA precursor. In this procedure, endogenous leukocyte RNA synthesis was allowed to fall to a baseline level prior to the addition of PHA. At forty-two hours, 100 micrograms of PHA-M was added, and twenty-four hours later one microcurie 8H-uridine (4.4 c./mM) was added; and, after a subsequent two hour incorporation
Fig. 1.—Incorporation of $^{3}{}^{14}{}^{C}$ thymidine into DNA of PHA stimulated lymphocytes. Marked reductions of thymidine uptake are observed in multiple myeloma and macroglobulinemia. The shaded area represents the 95 per cent confidence limits on the standard error of the mean.

Effect of Myeloma Plasma on Lymphocyte Response

Leukocytes from normal donors were isolated and washed as described above and cultured in MEM containing either 10 per cent autologous plasma, bovine serum, or cell free heparinized plasma from untreated myeloma patients whose cells had been studied in the DNA assay system. Plasma specimens were kept frozen at $-20\,^{\circ}{\text{C}}$ from the time of collection and thawed and brought to 37 $^{\circ}{\text{C}}$ one hour prior to the preparation of the cell cultures. The DNA assay was utilized as the indicator system to screen for inhibitory or stimulatory effects of myeloma plasma on normal lymphocytes cultured in the presence of phytohemagglutinin.

Immunoglobulin Characterization and Quantitation

The serum and urinary paraproteins of patients with myeloma and macroglobulinemia were characterized as to immunoglobulin class and light chain type by immunoelectrophoresis and immunodiffusion with monospecific rabbit antisera. Paraproteins were isolated by zone electrophoresis, DEAE cellulose and gel filtration chromatography on "Sephadex C-200" (Pharmacia Chemicals, Piscataway, N.J.) for further characterization. Determination of subclasses of the isolated $\gamma_{G}$-myeloma paraproteins was accomplished by several technics including immunodiffusion with specific antisera, as well as genetic typing of the isolated proteins with the Gm. system. Quantitation of the serum myeloma proteins was done by “Microzone” cellulose acetate electrophoresis (Beckman Instruments, Palo Alto, Calif.) and quantitative densitometry, with the total protein level determined by refractometry (American Optical Co., Keene, N.H.). Residual normal immunoglobulins of the nonparaprotein classes were quantitated by a modification of the Mancini radial gel diffusion technic.
Major infections were defined as bacterial infections that seriously compromised a vital organ or system, and were associated with signs of systemic toxicity such as fever, leukocytosis and tachycardia. History of past infection was tabulated prior to the determinations of lymphocyte nucleic acid synthesis or serum immunoglobulin levels.

**RESULTS**

**Incorporation of Thymidine into DNA**

The peripheral lymphocytes from the 20 myeloma patients and six patients with macroglobulinemia all demonstrated marked decreases in thymidine incorporation into DNA, with a mean of about 17 per cent of the controls. Uptake, as expressed as nucleotide incorporation (c.p.m./10⁶ lymphocytes) was 230 ± 37 (S.E.M.) in myeloma, 232 c.p.m. ± 67 in macroglobulinemia as compared to 1347 c.p.m. ± 157 in 17 normal controls, and 1629 c.p.m. ± 168 in 10 patients with diffuse hypergammaglobulinemia. The differences between the patient groups with myeloma and macroglobulinemia and the two control groups were highly significant (P < .001). The average thymidine uptake was higher in the myeloma patients who were not receiving chemotherapy (269 c.p.m.) than in the 5 who were receiving melphalan or corticosteroids (103 c.p.m.). Excluding those patients receiving chemotherapy, the circulating lymphocyte population in myeloma patients still had a highly significant impairment in thymidine incorporation compared to the control groups. Myeloma plasma of varying paraprotein types, when incubated with normal lymphocytes (Fig. 2) did not significantly alter the incorporation of thymidine into DNA.

**Incorporation of Uridine into RNA**

The peripheral lymphocytes from 17 patients with myeloma, four of whom were receiving chemotherapy, and 6 patients with macroglobulinemia were investigated (Fig. 3). The incorporation of ³H-uridine into lymphocytes from patients with myeloma was only approximately 33 per cent (214 c.p.m. ± 38) of that seen in normals (679 c.p.m. ± 150), while the uptake of uridine by macroglobulinemic lymphocytes was about 21 per cent (143 c.p.m. ± 67)
Fig. 3.—Incorporation of 3H-uridine into RNA of PHA stimulated lymphocytes. Marked reductions of uridine uptake are seen in the paraproteinemias.

of the control levels. The reduction of PHA stimulated lymphocyte RNA synthesis in these patient groups was highly significant with $P < .001$. Although four of the myeloma patients studied were receiving chemotherapy (Table 1) their lymphocyte response range was similar to that seen in the untreated patients. There was a wider range of uridine uptake in the benign hypergamaglobulinemic patient group, and an approximately 40 per cent higher mean incorporation of uridine into RNA (957 c.p.m. ± 124) than in the normals ($P < .05$).

Paraproteins, Normal Immunoglobulins and Lymphocyte Response

Serum for immunoglobulin typing and quantitation was obtained on all 26 patients with malignant dysproteinemia who were studied with the lymphocyte assays. In the myeloma patient group, 17 of 20 (75 per cent) had serum paraprotein peaks, of which 71 per cent were $\gamma G$ and 29 per cent were $\gamma A$ myeloma proteins. The kappa light chain determinant was present in 77 per cent of the proteins, and the remainder lambda. Two of the 3 patients who lacked serum "M" components had Bence-Jones proteinuria, one kappa and the other lambda. The remaining patient who lacked a detectable protein abnormality had marrow plasmacytosis and a lytic lesion.
which on biopsy was a plasmacytoma. Of the 12 γG myeloma proteins, the heavy chain subclasses (γG1, γG2, γG3 and γG4) had a frequency that might be expected in an average myeloma population. None were either γG4 or γD myeloma proteins. One of the γG myeloma patients (pt. 16) also had a γM paraprotein of type kappa, but had the clinical manifestations of the myeloma group.

Paraproteins of all 6 macroglobulinemia patients were isolated for light chain typing, and all were of type kappa. In one of the macroglobulinemic patients there was a diffuse increase in the levels of γG and γA globulins.

Serum paraprotein types, and quantitation, as well as levels of the residual normal immunoglobulin classes are summarized in Table 1. As has been noted previously, levels of the residual normal immunoglobulins are frequently reduced in myeloma and macroglobulinemia. This was also observed in the current study; 90 per cent of the patients with myeloma and 50 per cent of the patients with macroglobulinemia had significant reduction (> 2 S.D.) of one or more of the normal immunoglobulin components measured. The residual immunoglobulin defect is both quantitatively and qualitatively more severe in myeloma with 60 per cent of the patients having significant reductions in the levels of two of the immunoglobulin classes, whereas none of the 6 macroglobulinemic patients had a deficiency in both IgG and IgA immunoglobulins.

Residual normal immunoglobulin levels were reduced in almost all malignant dysproteinemic patients and the cellular uptake of thymidine and uridine was reduced in all the PHA cultures from the same subjects. There was no correlation between the degree of suppression in each system. In fact, the lymphocyte defect appeared to be equally severe in the macroglobulinemic patients who had higher average immunoglobulin levels (even excluding patient 26) than the myeloma patients. Two patients with myeloma with solitary lytic plasmacytomas in the spine without osteoporosis (patients 1 and 20) also had residual immunoglobulin levels within the normal range. There was no apparent relationship between the type or quantity of the paraprotein and the amount of precursor incorporation into DNA (Fig. 4) or RNA of lymphocytes stimulated by PHA.

Infection, Immunoglobulin Deficiency and Impaired DNA Synthesis

Major infections occurred in 8 patients with multiple myeloma within a period of 6 months prior to the lymphocyte studies. The infections which the patients had were either bacterial pneumonias or acute purulent bronchitis, and required antibiotic therapy for resolution. All of the patients with a history of infection had significant immunoglobulin deficiencies (Table 1). An additional 10 patients with myeloma had reduced immunoglobulin levels without a history of infection. In attempting to correlate the results of the lymphocyte studies with susceptibility to infection, it appeared that an association could be drawn between impaired lymphocyte DNA synthesis and major infection in myeloma patients with immunoglobulin deficiencies. For the purpose of this analysis, the 4 patients who were receiving chemotherapy were excluded because of reductions in uptake of thymidine which could have
### Table 1.—Patient Characterization, Immunoquantitation and Lymphocyte Response in Myeloma and Macroglobulinemia

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<tr>
<th>Diagnosis</th>
<th>Paraprotein Type and Level mgm. %</th>
<th>Residual Normal Immunoglobulin Levels mgm. %</th>
<th>In Vitro Lymphocyte Response to PHA CFM/10⁶ Lymphocytes</th>
<th>Chemotherapy mg./day</th>
<th>History of Bacterial Infection</th>
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<td>1. V.K.</td>
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<td>19. A.R.</td>
<td>λ BJ</td>
<td>&gt;2 Gm./24 hrs.</td>
<td>880</td>
<td>37</td>
<td>25</td>
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| 20. W.R. |   | no paraprotein | 960 | 135 | 46 | 97 | 23 | none |

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<td>510</td>
<td>140</td>
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* γMK—a second paraprotein in this patient with "Dielonal gammopathy."
† "Polyclonal" γG with both light chain types K and λ.
Patient 20 eventually developed a γG paraprotein.
resulted from treatment (as described above). Within the remaining restricted group of 14 untreated patients with immunoglobulin deficiency, the mean incorporation of thymidine into DNA was significantly lower (P < .05) in the 6 patients with a history of major infection (171 c.p.m. ± 97) than in the 8 patients who lacked such a history (376 c.p.m. ± 56).

**DISCUSSION**

The present study demonstrates an abnormality of nucleic acid synthesis of the PHA stimulated lymphocyte in myeloma and macroglobulinemia. Since myeloma plasma did not appear to impair normal lymphocyte function during the culture period, it would appear that the abnormality in the lymphocyte is not the result of the presence of the paraprotein or other plasma factors. The nucleic acid changes, whether specific or nonspecific indicators of alterations in lymphocyte function, nonetheless reflect a cellular abnormality in the circulating lymphocyte pool.

The abnormality noted in PHA stimulated lymphocytes in these two diseases is compatible with several alternative hypotheses:

1. The lymphocyte population with the potential to respond to mitogenic stimulation may be quantitatively reduced in cell number as compared to the number of “potentially responsive cells” in the normal individual.
2. The peripheral lymphocyte population responsive to mitogen may be qualitatively “defective” or “damaged,” and capable of making only an effete response to mitogenic (or antigenic) stimulation.
3. There may be a normal absolute number of circulating PHA responsive cells and an increased number of unresponsive cells with the morphology
of lymphocytes. This would result in a relative rather than an absolute decrease in responsive cells. If the unresponsive cells had the ability to bind or inactivate the "mitogenic message," they might interfere quantitatively with the in vivo lymphocyte response as well.

In vivo studies which have demonstrated reduced response to antigenic stimulation, and decreased synthesis rates of normal immunoglobulins in myeloma, as well as the reduced immunoglobulin levels described here and by others, are compatible with any of the above hypotheses.

The majority of experimental evidence suggests that in vivo the plasma cell arises morphogenetically from a small lymphocyte precursor, presumably through transitional stages involving large lymphocytes and undifferentiated "blast cells." As originally observed by Baney, Vazquez and Dixon, the development of antibody-producing plasma cells requires an intervening mitotic division of antigen-stimulated precursor cells. Several lines of evidence, summarized elsewhere (e.g.,), suggest that the PHA-stimulated cell and the most primitive cell in the plasma cell series may be related. In the patient population studied by Solomon et al., bone marrow aspirates showed a spectrum of immunoglobulin synthesizing cells ranging from small lymphocytes to mature plasma cells. A similar cell spectrum was seen in 4 of our patients in whom appropriate immunofluorescent studies were performed.

It is now well recognized that the lymphocyte plays a central role in the normal immune response. The early metabolic and biosynthetic changes in the lymphocyte which are associated with the response to mitogenic and antigenic stimulation have been extensively studied. Synthesis of DNA and cellular proliferation appear to be necessary steps which lead to the synthesis of specific antibodies after a secondary challenge with antigen in vivo or in vitro. Indeed, the blockade or destruction of just those cells which synthesize DNA in response to antigen effectively prevents cell division, as well as the formation of specific antibody. Further cell proliferation may not be necessary for continuation of immunoglobulin synthesis once an antibody response has been established.

Inasmuch as transformation of lymphocytes has been observed after exposure to antigens which typically evoke a serum antibody response such as diphtheria toxoid, as well as with PPD, which is characteristically associated with delayed hypersensitivity phenomena, the observation of lymphocyte transformation by morphologic or present biochemical means does not distinguish between subpopulations of immunocompetent cells. A delayed in vitro proliferative response to PHA has recently been reported by Rubin et al. as an explanation for the reduced responsiveness of the CLL lymphocyte to PHA; similar studies remain to be done in myeloma.

The findings in the present study suggest that there is an association, although they do not prove a causal relationship, between the inability of the lymphocyte to respond to PHA, reduced levels of normal immunoglobulin components, and susceptibility to infection.

It is possible that a cellular aberration in the lymphocyte may play a significant role in the pathogenesis of the plasma cell malignancies. A defect in a precursor cell common to both lymphocytes and plasma cells might
result either in aberrant lymphocytes (unable to respond to antigenic stimulation), in long-lived paraprotein producing plasma cells, or both. Such an underlying defect, if present, could possibly provide a partial explanation for both the deficiencies of normal immunoglobulins as well as the production of paraproteins. Alternatively, the lymphocyte abnormality may be a cellular sequela of the disease. In any event, the present studies provide evidence of a lymphocyte abnormality; whether this is cause or effect or merely coincidental is yet to be proven.

SUMMARY

Twenty-six patients with multiple myeloma and macroglobulinemia of Waldenström were studied clinically and immunologically with characterization of their paraproteins and normal immunoglobulins, as well as by in vitro culture of their peripheral lymphocytes for evaluation of DNA and RNA synthesis after phytohemagglutinin stimulation. The lymphocytes of the patients were found to be significantly deficient in response to PHA as compared to normals and patients with benign hypergammaglobulinemia. Levels of normal immunoglobulins were reduced in almost all of the paraproteinemic patients, but there was not a direct correlation between lymphocyte unresponsiveness and immunoglobulin deficiency. The defect in lymphocyte function appeared to be cellular inasmuch as normal lymphocytes had normal DNA synthesis when cultured in myeloma plasma. The decrease in lymphocyte nucleic acid synthesis appeared to be unrelated to immunoglobulin class, quantitative levels or antigenic characteristics of the patients’ paraproteins. Untreated myeloma patients with a past history of infection had lower levels of lymphocyte DNA synthesis than those patients who lacked such a history, suggesting a relationship between the in vitro lymphocyte response to PHA and the in vivo response to the antigenic challenge of bacterial infection.

SUMMARIO IN INTERLINGUA

Vinti-six patientes con myeloma multiplice e macroglobulinemia de Waldenström esseva studiate dinica- e immunologicamente con characterisation de lor paraproteinas e normal immunoglobulinas e etiam con cultivation in vitro de lor lymphocytos peripheric pro evaluatar lor synthese de acido desoxyribonucleic e acido ribonucleic post stimulation phytohemagglutininic. Le lymphocytos del patientes se trovava significativemente defective in lor responsa a phytohemagglutininia in comparation con illos de subjectos normal e de patientes con benign hypergammaglobulinemia. Le nivellos del immunoglobulinas normal esseva reduce in quasi omne le patientes paraproteinemic, sed il non existeva un correlation directe inter le nonresponsivitate lymphocytic e le carentia de immunoglobulinia. Le effecto in le function lymphocytic pareva esser cellular in tanto que normal lymphocytos habeva un normal synthese de acido desoxyribonucleic quando illos esseva culturate in plasma myelomatic. Le declino in le synthese lymphocytic de acido nucleic pareva esser nonrelacionate al classe immunoglobulinic, a nivellos quantitative o al characteristics antigene del paraproteinas del patientes. Nontractate patientes myelomatic con antecedentes de infection habeva plus basse nivellos del synthese lymphocytic de acido desoxyribonucleic que le patientes qui non habeva tal antecedentes. Isto suggestiona un relation inter le responsa del lymphocytos in vitro a phytohemaggglutininia e le responsa in vivo al provocation antigenic per un infection bacterial.

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Abnormal Nucleic Acid Metabolism of Lymphocytes in Plasma Cell Myeloma and Macroglobulinemia

SYDNEY E. SALMON and H. HUGH FUDENBERG