JCI The Journal of Clinical Investigation

Studies on the Pathogenesis of an Immune Defect in Multiple Myeloma

Teresa Paglieroni, Malcolm R. MacKenzie

J Clin Invest. 1977;59(6):1120-1133. https://doi.org/10.1172/JCI108736.

Research Article

The reduced capacity of patients with multiple myeloma to respond to antigen challenge is well recognized. Response to antigen involves antigen recognition, cell proliferation, and synthesis and secretion of antibody. This study examines this sequence of events in peripheral blood lymphocytes from untreated and treated patients with myeloma, from individuals with benign monoclonal gammopathy, and from normal healthy donors. Antigen-binding capacity was assessed by testing the ability of lymphocytes to bind radio-labeled pneumococcal polysaccharide, tetanus toxoid, or diptheria toxin. The in vitro proliferative response to these antigens as well as to pokeweed mitogen and streptokinase-streptodornase was evaluated. The secretion of immunoglobulin in response to pneumococcal polysaccharide, tetanus toxoid, and pokeweed mitogen by 2-4 × 10^6 lymphocytes in 7-day cultures was determined. The effects of coculture of myeloma peripheral blood lymphocytes and normal peripheral blood lymphocytes on immunoglobulin production and mixed leukocyte reactions were explored. All myeloma patients had normal numbers (3-8/5,000 cells) of antigen-binding cells. However, most showed a diminished antigen-induced blast transformation as measured by uptake of [^{125}I]5-iodo-2′-deoxyuridine in culture. Immunoglobulin production in response to specific antigen in myeloma lymphocytes was 30-80% less than in normal lymphocytes. Immunoglobulin synthesis and mixed leukocyte responses by normal peripheral blood lymphocytes could be suppressed by myeloma lymphocytes. Multiple suppressor populations were present. Thus, the immune defect in myeloma is beyond the antigen […]

Studies on the Pathogenesis of an Immune Defect in Multiple Myeloma

TERESA PAGLIERONI and MALCOLM R. MACKENZIE

From the Department of Internal Medicine, Section of Hematology and Oncology, University of California at Davis School of Medicine, Davis, California 95616

ABSTRACT The reduced capacity of patients with multiple myeloma to respond to antigen challenge is well recognized. Response to antigen involves antigen recognition, cell proliferation, and synthesis and secretion of antibody. This study examines this sequence of events in peripheral blood lymphocytes from untreated and treated patients with myeloma, from individuals with benign monoclonal gammopathy, and from normal healthy donors. Antigen-binding capacity was assessed by testing the ability of lymphocytes to bind radio-labeled pneumococcal polysaccharide, tetanus toxoid, or diptheria toxin. The in vitro proliferative response to these antigens as well as to pokeweed mitogen and streptokinase-streptodornase was evaluated. The secretion of immunoglobulin in response to pneumococcal polysaccharide, tetanus toxoid, and pokeweed mitogen by $2-4 \times 10^6$ lymphocytes in 7-day cultures was determined. The effects of coculture of myeloma peripheral blood lymphocytes and normal peripheral blood lymphocytes on immunoglobulin production and mixed leukocyte reactions were explored. All myeloma patients had normal numbers (3–8/5,000 cells) of antigen-binding cells. However, most showed a diminished antigen-induced blast transformation as measured by uptake of [^{125}I]5-iodo-2′-deoxyuridine in culture. Immunoglobulin production in response to specific antigen in myeloma lymphocytes was 30–80% less than in normal lymphocytes. Immunoglobulin synthesis and mixed leukocyte responses by normal peripheral blood lymphocytes could be suppressed by myeloma lymphocytes. Multiple suppressor populations were present. Thus, the immune defect in myeloma is beyond the antigen recognition step and involves both the proliferation of antigen-sensitive cells and immunoglobulin production. Further suppressive effects are imposed on normal cells, implying defects in immunoregulation in this disease.

Received for publication 26 April 1976 and in revised form 4 February 1977.

INTRODUCTION

Multiple myeloma is a disease characterized by malignant proliferation of plasma cells resulting in bone marrow replacement, osteolytic bone lesions, and paraprotein production. Despite the increased paraprotein production seen in many patients with multiple myeloma, there is impairment of polyclonal immunoglobulin synthesis as demonstrated both functionally by decreased capacity to form specific antibody, and clinically by increased susceptibility to certain bacterial infections (1). Lack of antigen-binding cells (ABC),[1] faulty expansion of the antigen-triggered immunocyte pool, increased catabolism of immunoglobulin, presence of "chalones" affecting maturation of bone marrow-derived (B) lymphocyte immunoglobulin synthesizing capacity, or presence of suppressor cells affecting B-cell maturation could all contribute to the observed immunoglobulin synthesis impairment (2–5). Data to be presented suggest that in patients with multiple myeloma, there are sufficient numbers of ABC that circulating lymphocytes have reduced capacity to undergo transition to immunoglobulin-secreting cells when exposed to pokeweed mitogen and certain antigens, that a suppressor cell population contributes to decreased immunoglobulin production, and that suppressor cell populations for other cell

[1] *Abbreviations used in this paper*: ABC, antigen-binding cells; BMG, benign monoclonal gammopathy; BSA, bovine serum albumin; EAC-RFL, lymphocytes forming rosettes with sheep erythrocytes coated with IgM anti-sheep erythrocyte antibody and C3 from human serum; EA-RFC, cells forming rosettes with human type O Rh-positive erythrocytes coated with human anti-D immunoglobulin; E-RFL, lymphocytes forming rosettes with sheep erythrocytes; [^{125}I]UdR, [^{125}I]5-iodo-2′-deoxyuridine; MLC, mixed leukocyte culture; MMC, mitomycin C: PBL, peripheral blood leukocytes, PBS, phosphate-buffered saline; PHA-M, phytohemagglutinin-M; PP, pneumococcal polysaccharide III; PWM, pokeweed mitogen; SIg, surface immunoglobulin-bearing cell; SKSD, streptokinase-streptodornase; SRBC, sheep erythrocytes; TT, tetanus toxoid.

functions may play a role in this disease. Such observations are significant in relation to approaches to therapy in this disease.

METHODS

Patient selection. 30 different healthy volunteer donors between 22 and 69 yr of age who were receiving no medication were used as controls. A total of 32 different patients with multiple myeloma between 55 and 89 yr of age (9 of these patients were untreated at the time of study) and 15 different patients with benign monoclonal gammopathy were included in these studies. The diagnosis of multiple myeloma was based on at least two of the following findings: (*a*) discovery of plasma cells constituting more than 20% of the total nucleated cellularity of aspirated bone marrow without other known cause, (*b*) presence of a monoclonal serum or urine gamma globulin peak on electrophoresis in combination with a reduction of other serum immunoglobulins, and (*c*) presence of bony lesions in the skull, vertebral column, or pelvic bones. Patients with multiple myeloma were treated with intermittent dosages of alkylating agents and corticosteroids, and were sampled 4–6 wk after their last drug cycle. All multiple myeloma patients tested had IgA or IgG M components; none had macroglobulinemia or light-chain disease. Patients were diagnosed as having benign monoclonal gammopathy if their serum gamma globulin peak was less then 3 g/dl and if other immunoglobulin levels were within normal limits. There was no evidence of anemia, renal disease, or skeletal lesions. These patients were asymptomatic, and their serum gamma globulin levels were stable for at least 3 mo.

Isolation of peripheral blood lymphocytes. Lymphocytes were separated from fresh heparinized blood (20 U preservative-free heparin/ml of blood) obtained by venipuncture on Ficoll-Hypaque density gradients (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) using a modification of the method of Böyum (6). Contaminating erythrocytes were lysed by resuspending the Ficoll-Hypaque interface layer in 0.83% ammonium chloride for 7 min before washing the cells three times in phosphate-buffered saline (PBS, 0.15 M NaCl in 0.01 M Na_2PO_4, pH 7.2–7.4). Preparations were 85–95% viable as determined by trypan blue dye exclusion and were only used if greater than 70% of the lymphocytes present in the original specimen were recovered. Lymphocyte preparations were depleted of phagocytic mononuclear cells by incubating 50 ml of blood with 250 mg of sterile carbonyl-iron powder (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.) at 37°C in 5% CO_2 for 30–60 min with gentle continuous agitation. Iron-filled cells were removed by differential centrifugation through Ficoll-Hypaque density gradients. This method resulted in lymphocyte populations containing less than 1% monocytes as determined by nonspecific esterase staining (7) and latex particle ingestion.

Depletion of lymphocyte subpopulations. Populations enriched for B cells were obtained by removing sheep erythrocyte receptor-bearing lymphocytes (E-RFL) on Ficoll-Hypaque density gradients (8). Several 10 × 75-mm plastic tubes, each containing 0.4 ml of a 3–5 × 10^6 lymphocyte/ml suspension, were incubated with an equal volume of 0.5% sheep erythrocytes (SRBC) for 5 min at 37°C. Mixtures were centrifuged at 200 *g* for 5 min, then incubated at 4°C for 4 h. The erythrocyte/lymphocyte pellets were gently dispersed, pooled until a 3-ml volume was obtained, and then layered onto a Ficoll-Hypaque mixture consisting of three parts 9% Ficoll and two parts 35% Hypaque. After centrifugation at 400 *g* for 40 min, cells were removed from the interface layer and again carried through the above procedure. E-RFL were found in the pellet. A lymphocyte subpopulation enriched for B cells remained at the Ficoll-Hypaque mixture interface. Less than 1% E-RFL remained in the interface layer.

Populations enriched for thymus-derived (T) cells were obtained using three different methods. (*a*) Surface immunoglobulin-positive lymphocytes were removed from Ficoll-Hypaque lymphocyte preparations by passage through nylon fiber columns using methods described by Greaves and Brown (9). Briefly, 300 mg of sterile washed nylon fibers (Nylon fiber LP 1 Leuko-pack, Fenwal Inc., Ashland, Mass.) were packed in 5-ml sterile plastic syringes. Columns were equilibrated at 37°C with RPMI 1640 supplemented with 2 mM glutamine, 15% decomplemented-pooled gamma-globulin-free AB serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Viable lymphocytes (5 × 10^7 in 1 ml) were added to the equilibrated columns. Lymphocytes were eluted with 10 ml of supplemented RPMI 1640 at a rate of 2 ml/min. Approximately 50% of the cells applied to the columns were recovered. Viability was 90% or greater. Eluted cell subpopulations contained less then 2% immunoglobulin-positive lymphocytes as judged by standard immunofluorescence methods (10) and less than 1% monocytic cells as judged by nonspecific esterase stains and latex particle ingestion. (*b*) Cells forming rosettes with human type O Rh-positive erythrocytes coated with human anti-D immunoglobulin were removed from Ficoll-Hypaque-purified monocyte-depleted lymphocyte preparations by methods previously described (11). Ficoll-Hypaque-purified monocyte-depleted peripheral blood leukocytes (PBL) (20 × 10^6 cells) in 1 ml of RPMI 1640 supplemented as above were mixed with an equal volume of a 1.0% suspension of human type O Rh-positive cells sensitized for 30 min at 37°C with human anti-D. Cells were centrifuged at 200 *g* for 5 min and then were incubated at room temperature for 30 min. The pellet was gently resuspended, then layered on a Ficoll-Hypaque gradient prepared as described above. Gradients were centrifuged for 40 min at 400 *g*. EA-RFC were present in the pellet. Lymphocytes containing less than 1% EA-RFC remained at the Ficoll-Hypaque mixture interface. (*c*) Complement receptor-bearing lymphocytes (EAC-RFL) were removed from Ficoll-Hypaque-purified monocyte-depleted lymphocyte preparations by methods similar to those described above. Monocyte-depleted lymphocyte preparations (3–5 × 10^6 cells/ml) were mixed at 37°C for 30 min with an equal volume of a 0.5% suspension of erythrocytes prepared as follows: immunoelectrophoretically pure rabbit anti-sheep erythrocyte IgM was titrated to one half of the minimum agglutinating dose in Hanks' balanced salts solution. Approximately 0.1 ml of packed washed SRBC less than 1 wk old was incubated with an equal volume of the appropriate dilution of IgM for 30 min at 37°C and for 15 min at 4°C. The antibody-coated cells were washed three times in normal saline, then incubated with a 1:10 dilution of fresh human serum absorbed at 37 and 4°C with washed SRBC. The antibody- and complement-coated cells were washed three times in PBS, then resuspended to 0.5% in Hanks' balanced salts solution warmed to 37°C. As above, cells were layered on a Ficoll-Hypaque density gradient and centrifuged at 400 *g* for 40 min. EAC-RFL were present in the pellet. The SRBC could be dissociated from the lymphocytes by treatment with a 1:10 dilution of goat anti-human C3 for 30 min. Cells present in the interface layer contained less than 2% EAC-RFL. Since most EAC-RFL from normal patients also bear surface immunoglobulin and would be removed by passage over nylon

fiber columns, the EAC-RFL-depletion technique was used on peripheral blood lymphocyte populations from myeloma patients who had an abnormal population of EAC-RFL which did not simultaneously bear surface immunoglobulin (12).

Antisera. Antisera coated with fluoroscein (rabbit anti-human IgG, IgM, IgA, and polyvalent anti-immunoglobulin) were purchased from Meloy Laboratories Inc. (Springfield, Va.). Conjugated antisera with fluorescein to protein ratios between two and six were run through Sephadex G-25 columns (Pharmacia Fine Chemicals Inc.) equilibrated with PBS before use. Unused conjugates were frozen in small aliquots at a protein concentration of 2 mg/ml or greater for later use. In addition, IgG, rabbit anti-human IgG, rabbit anti-human IgM, and goat anti-rabbit immunoglobulin were purchased from Meloy Laboratories Inc. A crucial aspect of this study was the antisera specificity. Reagents were tested by gel double diffusion and immunoelectrophoresis of the antisera against human serum undiluted and diluted 1:4. Antisera was shown to agglutinate erythrocytes coated with purified immunoglobulin of the homologous class but no other. In blocking experiments, purified immunoglobulin of the homologous class would inhibit the action of the antisera. To rule out the possibility that the antisera were contaminated with light chain antibody, antisera were tested before and after absorption with human light chains (Bench Jones kappa and lambda type, Meloy Laboratories, Inc.). No differences in results were found before and after absorption with human light chains.

Antigens. Partially purified diphtheria toxin, tetanus toxoid (TT), pneumococcal polysaccharide III (PP), and purified protein derivative (Parke, Davis & Company, Detroit, Mich.) were obtained. Diptheria toxin, TT or bovine serum albumin (BSA) (60 μg) was labeled with 2.5 mCi ^{125}I using 20 μg chloramine T at pH 7.3 (13). PP internally labeled with ^{14}C was a gift. All antigens, both radiolabeled and unlabeled, were dialyzed extensively against saline solutions before use.

Antigen-binding cells. The technique for determination of ABC was a modification of the method described by Liburd and McPherson (14). Varying concentrations of antigen, ranging from 10 to 80 ng, were added to 1×10^6 Ficoll-Hypaque-purified lymphocytes in 1 ml of RPMI 1640 supplemented with 15% decomplemented gamma globulin-free AB serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (Gibco Diagnostics, Grand Island Biological Co., Grand Island, N. Y.) in 12 × 75-mm plastic tubes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.). Preliminary experiments showed that 80 ng of antigen was optimal for the detection of ABC and results shown are from experiments using this antigen concentration. After a 30-min incubation at 4°C, tubes were centrifuged at 2,000 *g* for 15 min at 4°C. The supernate was decanted, and the cells were washed three more times in 3-ml volumes of RPMI 1640. Cell pellets containing ^{125}I-labeled antigen were counted directly in an automatic Searle gamma counter, model 1185 (Searle Analytic Inc., Des Plaines, Ill.). Cell pellets containing ^{14}C-labeled antigen were first solubilized in Protosol/toluene 1:3 (New England Nuclear, Boston, Mass.), then counted on a Beckman LS-100 C beta counter (Beckman Instruments, Fullerton, Calif.) in 10 ml of Omnifluor (New England Nuclear). The amount of radioactivity bound per cell was correlated with cell-bound antigen as determined by autoradiography (12). For example, the binding of 19,900 counts of a particular batch of [^{125}I]TT corresponded to 10 ABC/5,000 lymphocytes as determined by autoradiography. The number of cells binding antigen were then calculated.

Mitogen and antigen stimulation. Ficoll-Hypaque-purified monocyte-depleted lymphocytes (1×10^5 cells) were incubated in 1 ml of RPMI 1640 supplemented with 2 mM glutamine, 20% decomplemented fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in tissue culture plates (3008 Multiwell tissue culture plates, Falcon Plastics) containing 10, 25, or 50 μl/culture phytohemagglutinin-M (PHA-M, Difco Laboratories, Detroit, Mich), 5, 10, or 20 μl/culture pokeweed mitogen (PWM, Gibco Diagnostics), 80 ng TT, 80 ng streptokinase-streptodornase (SKSD, Lederle Laboratories, Pearl River, N.Y.), 80 ng PP, 80 ng purified protein derivative, or 80 ng BSA for 4 days at 37°C in a 5% CO_2-humidified incubator. Control cultures consisted of lymphocytes incubated without mitogen or antigen. After 4 days in culture, cultures were pulsed with 1.0 μCi/culture [^{125}I]5-iodo-2′-deoxyuridine ([^{125}I]UdR), specific activity 1 Ci/mmol (Amersham/Searle Corp., Arlington Heights, Ill.) for 6 h before harvesting (15). Cells were harvested, washed, and then counted on an automatic Searle gamma counter, model 1185, for 1–10 min. Results were expressed as counts per minute.

Assay system for IgG and IgM produced in culture. IgG or IgM produced by Ficoll-Hypaque-purified monocyte-depleted lymphocyte preparations in culture was determined as described previously by Waldmann et al. (16). Briefly, 2×10^6 Ficoll-Hypaque-purified lymphocytes/ml in 2-ml cultures were incubated at 37°C in a 5% CO_2-humidified incubator in RPMI 1640 supplemented with 2 mM glutamine, 20% decomplemented fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in 13 × 100-mm plastic culture tubes (Falcon Plastics) in the presence or absence of optimal amounts of PWM, TT, or PP. After 7 days in culture, tubes were centrifuged at 2,500 rpm for 10 min. The amount of IgG or IgM synthesized and secreted into the culture supernate was determined by a double-antibody radioimmunoassay. Specificity of antisera used in these experiments was determined as described in the antisera section above. 1 mg of IgG or IgM in 1 ml of 0.05 M phosphate buffer, pH 7.0, was labeled with 2–3 mCi ^{125}I using a chloramine-T method. The dilution of anti-IgG or anti-IgM which would bind 60% of a suitable amount of labeled IgG or IgM was determined. Antigen-antibody dilution curves were constructed over an antigen range of 1.0–0.1 μg of labeled antigen and 1:10–1:500 dilution of antisera. The amount of goat anti-rabbit gamma globulin added to precipitate the rabbit immunoglobulin was twice the equivalence amount. In the actual assay, a dilution of culture supernate or standard (0.05 ml) was added to 0.05 ml of anti-IgG or anti-IgM for 3 h at room temperature. Then 0.05 ml of goat anti-rabbit immunoglobulin was added to the 10 × 75-mm siliconized glass tubes which were incubated for 15 h at 4°C. After overnight incubation, 1.0 ml of 0.01 M Tris buffer, pH 7.0–7.4, was added to each tube. Tubes were centrifuged at 4–6°C at 2,000 *g* for 20 min. The supernate was decanted and the pellet was counted. Standards were made up in 0.01 M Tris buffer, pH 7.4, with 20% normal rabbit serum. The binding of labeled immunoglobulin could be inhibited by cold specific immunoglobulin up to 90%. When known amounts of IgG or IgM were added to appropriate culture supernates, 90–95% of the added IgG or IgM was detected.

Co-culture experiments. To determine the presence or absence of circulating suppressor cells, a coculture technique similar to one previously described by Waldmann et al. (16) was used. Ficoll-Hypaque-purified, or Ficoll-Hypaque-purified monocyte-depleted cell populations from multiple myeloma patients, benign monoclonal gammopathy patients,

or allogeneic normal patients (4×10^6 cells in 2-ml cultures in RPMI 1640 supplemented as above) were incubated with equal concentrations of lymphocytes from normal control patients in the presence of 20 μl/culture PWM(the total culture volume was 4 ml). Preliminary experiments showed that in most cases, 20 μl/culture PWM produced maximal stimulation of immunoglobulin production. The expected IgG or IgM production in the absence of suppressor effects approximated the sum of IgG or IgM produced by each cell population cultured alone (4×10^6 cells/2 ml) in the presence of optimal amounts of PWM. Percent of expected Ig synthesis by cells in coculture = 100 × (Nanograms of Ig synthesized by 4×10^6 PBL from both subjects in coculture in the presence of PWM/sum of the nanograms of Ig synthesized by 4×10^6 PBL from each subject cultured separately in the presence of PWM). Percent suppression of Ig synthesis = 100 − percent of expected Ig synthesis by cells in co-culture.

Mitomycin C treatment of lymphocytes. Ficoll-Hypaque-purified lymphocytes ($5–10 \times 10^6$ cells) were incubated with 10 ml of RPMI 1640 containing 50 μg/ml of mitomycin C (MMC, Sigma Chemical Co., St. Louis, Mo.) for 30 min at 37°C. After the incubation, the cells were washed three times in PBS.

One-way mixed leukocyte culture. Viable Ficoll-Hypaque-purified but not monocyte-depleted mononuclear cell preparations from normal volunteers (1×10^6 cells/0.1 ml) were incubated with an equal number of MMC-treated allogeneic normal peripheral blood stimulator cells in triplicate cultures in wells of 3008 Multiwell tissue culture plates. Controls consisted of PBL incubated with autologous MMC-treated PBL. To test if suppressor cells affected the ability of a cell population to stimulate another unrelated cell population in one-way mixed leukocyte culture, viable MMC-treated PBL from multiple myeloma patients or benign monoclonal gammopathy patients were added in varying concentrations (0.5, 1, 2, and 4×10^6 cells /0.1 ml) to the above cultures. Cultures were incubated for 6 days, then they were pulsed for 6 h with [^{125}I]UdR (1 μCi/culture), harvested, and washed. The radioactivity in the harvested cells was determined by counting them in an automatic Searle gamma counter, model 1185 (17). Results were expressed in counts per minute. The percent suppression in the presence of stimulator cells was calculated as follows: percent expected counts = (cpm of one-way mixed leukocyte culture [MLC] in the presence of a "suppressor" cell population/cpm of one-way MLC in the absence of the suppressor cell population) × 100. Percent suppression = 100 − percent expected counts. If the percent suppression was a negative number, there was no suppression but stimulation instead. Percent suppression of 50% or greater was considered significant inhibition.

Inhibition of MLC by MLC supernates. Supernatant fluid was harvested from MLC of allogeneic normals and leukocytes manifesting suppressor activity. 1 ml of supernatant fluid was mixed with an equal volume of fresh media and used as the culture media for a standard allogeneic MLC reaction. A 50% or greater reduction in the expected cpm was considered significant inhibition.

Statistical analysis. In experiments where individual results were shown, data were expressed as mean counts per minute/culture±SEM for triplicate determinations. The statistical significance of the difference between mean values in the various groups was determined by Student's *t* test. The difference between means were considered significant whenever the *P* values were less than or equal to 0.05.

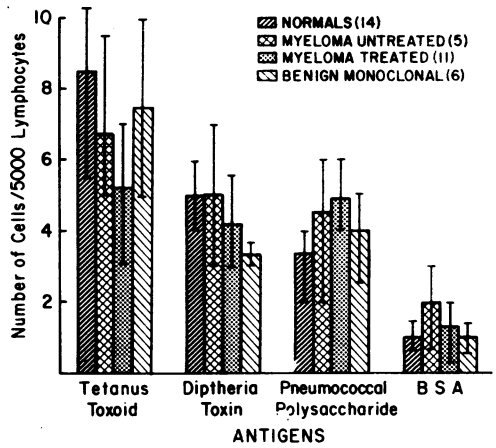

FIGURE 1 Antigen-binding cells in peripheral blood. Ficoll-Hypaque-purified PBL (1×10^6 cells) were incubated with the labeled antigens shown above. The amount of label taken up by the cells was correlated with the number of antigen-binding cells present. Results are expressed as the mean± 1 SD.

RESULTS

Demonstration of antigen-binding cells in the peripheral blood of patients with multiple myeloma. Fig. 1 shows the results of antigen-binding cell experiments. 14 normal patients tested ranged from 25–69 yr of age. All patients used in the calculations had been immunized against tetanus and diphtheria. PBL from one patient tested who had not been immunized against tetanus or diptheria had less than one antigen-binding cell per 5,000 lymphocytes. This patient's results were not included in the normal group mean. BSA was used in these experiments for baseline purposes. Less than 2 ABC/5000 lymphocytes were present in most patients tested. Of those tested, one untreated patient with multiple myeloma and three patients treated for myeloma had hospital documentation of pneumococcal pneumonia. When means were compared using Student's *t* test, the number of antigen-binding cells in the peripheral blood of normal patients did not differ at the 95% confidence level from the number of antigen-binding cells in the peripheral blood of patients treated for myeloma, those with myeloma who were untreated, or those with benign monoclonal gammopathy.

Stimulation of DNA synthesis in peripheral blood lymphocytes of multiple myeloma patients by nonspecific mitogens or specific antigens. Results in Fig. 2A show that the peripheral blood lymphocyte responses to PHA-M or PWM were normal or increased in untreated patients with multiple myeloma but that the PWM response was significantly decreased ($P < 0.01$) in patients treated for multiple myeloma.

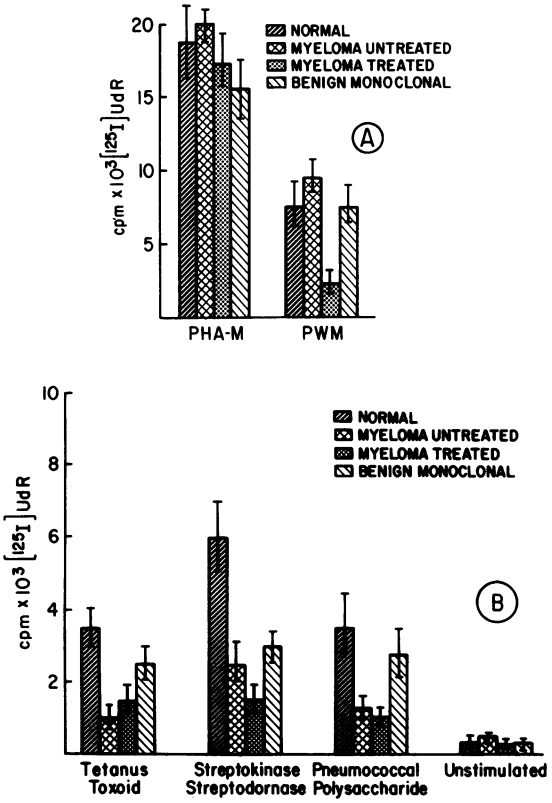

FIGURE 2 (A) Stimulation of PBL with nonspecific mitogens. Ficoll-Hypaque-purified PBL (1×10^5 cells) were incubated with 10 μl/culture PWM or 25 μl/culture PHA-M for 4 days as described in Methods. Tests were performed in triplicate. Results are expressed as the mean±1 SD of data from 20 normal, 5 untreated multiple myeloma, 13 treated multiple myeloma, and 7 BMG patients. (B) Stimulation of PBL by antigens. Ficoll-Hypaque-purified PBL (1×10^5 cells) were incubated with or without the appropriate amount of antigen for 4 days as described in Methods. Results represent the mean±1 SD of data from 20 normal, 5 untreated multiple myeloma, 11 treated multiple myeloma, and 5 BMG patients. All tests were performed in triplicate.

However, dose-response curves for treated multiple myeloma, untreated multiple myeloma, benign monoclonal gammopathy, and control patient groups were different. For example, DNA synthesis as measured by [^{125}I]UdR uptake after stimulation with 25 μl/culture PHA-M was similar in all groups tested. However, stimulation with greater concentrations of PHA-M (30–100 μl/culture) caused significantly decreased responses ($P < 0.025$) in both treated and untreated multiple myeloma patient Ficoll-Hypaque-purified monocyte-depleted lymphocyte populations when compared to responses in equivalent lymphocyte populations from normal control patients or patients with benign monoclonal gammopathy. The response of untreated myeloma patient PBL to low concentrations of PWM (2 μl/culture) was significantly greater than responses of normal PBL to low concentrations of PWM. At concentrations of 10 μl/culture PWM, responses of untreated multiple myeloma patient PBL were greater than or equal to normal PBL responses. At higher concentrations of PWM, the response curves for untreated multiple myeloma patients plateaued sooner than did the curves for normal control or benign monoclonal gammopathy patients. Lymphocytes from treated multiple myeloma patients were stimulated to a lesser degree than lymphocytes from untreated multiple myeloma patients, benign monoclonal gammopathy patients, or normal controls at all concentrations of PWM tested. These studies are presented in detail elsewhere (12, 18). All of these studies were performed using lymphocyte preparations depleted of monocytes by carbonyl-iron ingestion followed by Ficoll-Hypaque purification. If monocytes were not removed, we found significantly different results, i.e. PHA-M responses in untreated and treated multiple myeloma patients were slightly but significantly decreased as compared to normals.

The [^{125}I]UdR uptake of treated and untreated myeloma patient PBL in the presence of optimal amounts of SKSD, PP, or TT was slightly but significantly decreased ($P < 0.05$, Fig. 2B) when compared to PBL from benign monoclonal gammopathy patients or normal controls. PBL from patients with benign monoclonal gammopathy showed normal stimulation by TT and PP, but decreased stimulation by SKSD. Again, dose response curves for the various patient groups varied slightly but not significantly. Results shown are for only one antigen concentration.

In vitro immunoglobulin production. The in vitro production of IgG and IgM by peripheral blood lymphocytes after PWM or antigen stimulation was determined in 15 normal individuals. These results were contrasted with the nanograms of IgG produced in culture by PBL from untreated and treated IgA multiple myeloma and IgA benign monoclonal gammopathy patients (Fig. 3A) or with the nanograms of IgM produced in culture by PBL from untreated and treated IgG multiple myeloma and IgG benign monoclonal gammopathy patients (Fig. 3B). The production of immunoglobulin by all groups of multiple myeloma patients tested was significantly depressed ($P < 0.05$) when compared to immunoglobulin produced in vitro by PBL from normal and benign monoclonal gammopathy patients in response to the same mitogens or antigens. Note: these experiments were performed using lymphocyte preparations depleted of monocytes by carbonyl iron ingestion followed by Ficoll-Hypaque purification.

Suppression of in vitro [^{125}I]UdR uptake. Peripheral blood lymphocytes from 10 different normal patients were incubated in the presence of optimal

amounts of PWM, TT, and SKSD. In addition, an equal number of MMC-treated lymphocytes (from preparations which had not been monocyte depleted) from treated and untreated multiple myeloma patients, or patients with benign monoclonal gammopathy were added. Results are shown in Table I. Two out of four untreated multiple myeloma patients tested suppressed PWM responses of normal PBL, and four out of four untreated multiple myeloma patients tested suppressed normal PBL responses to TT and SKSD. In all four cases, suppression was significantly decreased in the absence of monocytes. No suppression was observed in any of the benign monoclonal gammopathy patients tested. Normal PBL responses were suppressed by only two out of seven treated multiple myeloma patients tested.

Suppression of in vitro immunoglobulin production. To determine if circulating suppressor cells could cause the decreased in vitro immunoglobulin production, coculture experiments were performed. When coculture experiments were done between 15 pairs of unrelated normal patients, the in vitro IgG or IgM synthesis suppression never exceeded 28%. PBL from 5 untreated multiple myeloma, 15 treated multiple myeloma, and 10 benign monoclonal gammopathy patients were cocultured with PBL from 15 different normal patients. A summary of the results obtained is shown in Fig. 4. In five untreated multiple myeloma patients tested, the percent suppression of the appropriate immunoglobulin measured varied from 38–65% in PWM-stimulated cultures, from 31–75% in TT-stimulated cultures, and from 47–82% in PP-stimulated cultures depending on which normal patient was used in the coculture experiment. In 15 treated myeloma patients tested, suppression of the appropriate immunoglobulin synthesis measured varied from 8–61% in PWM-stimulated cultures, from 1–75% in TT-stimulated cultures, and from 1–54% in PP-stimulated cultures. Only 6 out of 15 different patients treated for multiple myeloma who were tested demonstrated greater than 30% suppression of in vitro synthesis of the particular immunoglobulin tested. When PBL from 10 patients with benign monoclonal gammopathy were cocultured with PBL from different normal patients, suppression of the appropriate immunoglobulin production in the presence of PWM, TT, or PP was less than 15% except in the case of one IgG kappa benign monoclonal gammopathy (BMG) patient. This patient suppressed normal PBL IgM synthesis in the presence of PWM, TT, and PP; 41, 45, and 47%, respectively. If BMG patient PBL were incubated with treated or untreated myeloma PBL shown to suppress normal PBL in coculture, BMG patient PBL were also suppressed.

MMC treatment of multiple myeloma PBL before they were used in co-culture experiments only slightly

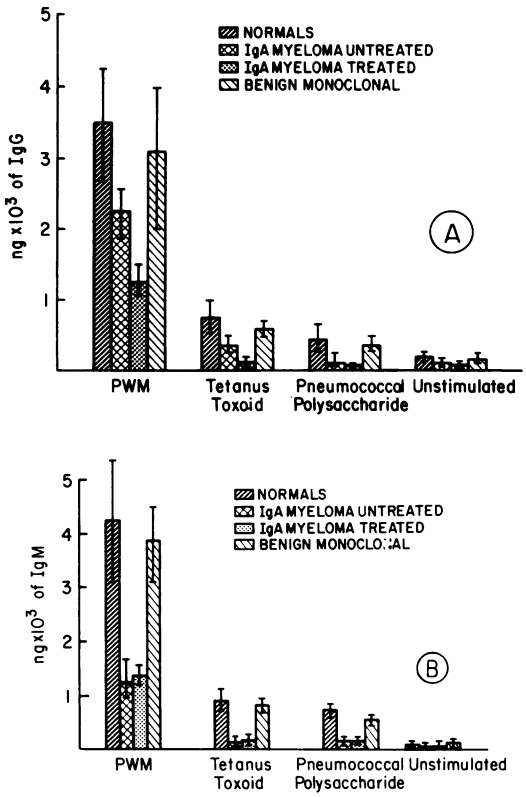

FIGURE 3 (A) Quantity of IgG produced and released into the supernate in 7-day cultures by 4×10^6 antigen- or mitogen-stimulated PBL. PBL from 20 normal, 2 untreated IgA multiple myeloma, 5 treated IgA multiple myeloma, and 4 IgA BMG patients were included in this study. Results represent the mean nanograms±1 SD of IgG produced and released in vitro. Experiments were performed in triplicate. (B) Quantity of IgM produced and released into the supernate in 7-day cultures by 4×10^6 antigen- or mitogen-stimulated PBL. PBL from 20 normal, 4 untreated IgG multiple myeloma, 14 treated IgG multiple myeloma, and 7 IgG BMG patients were included in this study. Results represent the mean nanograms±1 SD of IgM produced and released in vitro. Experiments were performed in triplicate.

decreased the suppressive effect observed in the above co-culture experiments. However, if cells were not viable, no suppressive effect could be demonstrated. Concanavalin A activation (50 μg/ml) of multiple myeloma PBL populations for 48 h before coculture with normal PBL enhanced the suppressive effect from 20–75%. Activation with either PHA-M or PWM before co-culture experiments had no particular enhancing effect. The ratio of myeloma patient PBL to normal PBL had to be 0.5–1 or greater for the suppressive effect to be observed. PBL from one untreated multiple myeloma patient tested were able to suppress responses of PBL from six different normal patients and four different BMG patients tested. No immunoglobulin synthesis suppression was observed when normal PBL were co-cultured with PBL

TABLE I

The Effect of MMC-Treated PBL from Treated and Untreated Multiple Myeloma Patients and BMG Patients on Mitogenic Responses of Normal Human Peripheral Blood Lymphocytes

Responding cells*	MMC-treated cells	[^{125}I]UdR incorporation in response to mitogens					
		PWM		TT		SKSD	
		cpm	Inhibition	cpm	Inhibition	cpm	Inhibition
			%		%		%
PBLN1	—	9,863±333	+54	4,116±286	+44	5,996±226	+63
	‡PBLMM1	4,486±306		2,313±156		2,226±451	
PBLN2	—	8,762±376	+43	3,763±185	+49	5,854±372	+61
	‡PBLMM2	5,013±387		1,911±163		2,286±201	
PBLN3	—	11,596±692	+8	4,096±222	+54	6,616±189	+65
	‡PBLMM3	10,662±589		1,896±236		2,292±161	
PBLN4	—	13,163±366	−6	4,233±269	+76	8,016±212	+73
	‡PBLMM4	13,986±382		1,008±303		2,203±206	
PBLN5	—	7,986±372	−34	3,166±226	−40	5,456±215	−41
	PBLBMG1	10,698±477		4,432±299		7,676±298	
PBLN6	—	9,959±356	−32	3,068±306	−31	6,063±303	−15
	PBLBMG2	13,163±779		4,013±511		6,998±414	
PBLN7	—	8,966±249	−34	3,383±449	−20	6,696±286	−3
	PBLBMG3	11,989±456		5,066±221		6,878±373	
PBLN8	—	9,963±443	−22	3,879±228	−28	6,369±309	−5
	PBLBMG4	12,161±806		4,963±269		6,662±217	
PBLN9	—	9,929±442	−30	3,636±259	−37	5,966±226	−16
	PBLBMG2	12,863±769		4,993±263		6,906±358	
PBLN10	—	9,896±703	−1	3,323±299	−34	6,062±401	−16
	PBLMM8	9,969±876		4,469±351		7,062±438	
PBLN1	—	9,863±333	0	4,116±286	−22	5,996±226	0
	PBLMM9	9,844±262		5,032±403		5,998±198	
PBLN2	—	8,762±376	−3	3,763±185	−5	5,854±372	−1
	PBLMM10	9,066±386		3,936±531		5,898±446	
PBLN4	—	13,163±366	−8	4,233±269	−17	8,016±212	−13
	PBLMM11	14,161±998		4,944±396		9,019±502	
PBLN5	—	7,986±372	+60	3,166±226	−25	5,456±215	+64
	PBLMM12	3,232±336		3,962±299		1,986±403	
PBLN7	—	8,966±249	+66	3,383±449	+14	6,696±286	+71
	PBLMM13	3,066±206		2,916±256		1,962±441	
PBLN8	—	9,963±443	−2	3,879±228	−5	6,369±309	−2
	PBLMM14	10,196±532		4,062±383		6,516±409	
PBLN9	—	9,929±442	−32	3,636±259	−36	5,966±226	−34
	PBLN1	13,142±766		4,946±444		7,997±463	
PBLN10	—	9,896±703	−43	3,323±299	−44	6.062±401	−34
	PBLN2	14,116±991		4,796±306		8,093±403	

* Normal donor PBL (1×10^5 cells) were mixed with 10 μl/culture PWM, 80 ng TT, or 80 ng SKSD/culture in the presence or absence of an equal number of MMC-treated allogeneic cells. PBL (1×10^5 cells) from untreated multiple myeloma, treated multiple myeloma, BMG, or allogeneic normal patients were treated with MMC and added to cultures of normal PBL stimulated with PWM, TT, or SKSD.

‡ Indicates PBL were from untreated multiple myeloma patients. Cultures were incubated 5 days and were then pulsed for 6 h with 1 μCi/culture [^{125}I]UdR. Results are expressed as mean counts per minute of triplicate cultures±the SE. Percent expected counts = (counts in presence of MMC-treated cells/counts in absence of MMC-treated cells) × 100. Percent inhibition = 100 − percent expected counts. Negative results implied stimulation.

from 10 different patients with chronic lymphocytic leukemia (2 of whom were untreated).

The suppressor cell. Experiments performed in the preceding section used Ficoll-Hypaque-purified PBL preparations from multiple myeloma patients which were monocyte depleted. Additional experiments were performed as above except PBL from multiple myeloma patients were not monocyte depleted. When five different normal PBL were mixed with PWM and Ficoll-Hypaque-purified monocyte-depleted preparations from five different untreated multiple myeloma patients, the observed suppression of either IgG or IgM in vitro immunoglobulin synthesis by stimulated normal PBL was 38, 49, 51, 68, and 69%, respectively (Fig. 4). When the same normal PBL were mixed with PWM and Ficoll-Hypaque-purified but not monocyte-depleted preparations from the same untreated multiple myeloma patients as above, the observed suppression of immunoglobulin synthesis was 95, 55, 52, 92, and 75%, respectively. Similarly, when normal PBL were mixed with PWM and monocyte-depleted PBL preparations from treated multiple myeloma patients, 6/15 treated multiple myeloma patients tested suppressed appropriate PWM-stimulated immunoglobulin synthesis by normal PBL greater than 30% (Fig. 4). However, when PBL preparations from these treated multiple myeloma patients were used which were not monocyte depleted, 8/15 patients showed 30% suppression or greater of the expected PWM-stimulated production of appropriate immunoglobulin by normal PBL. These results differ from results reported by Broder et al. (5), and the results suggest that a significant suppressive response is

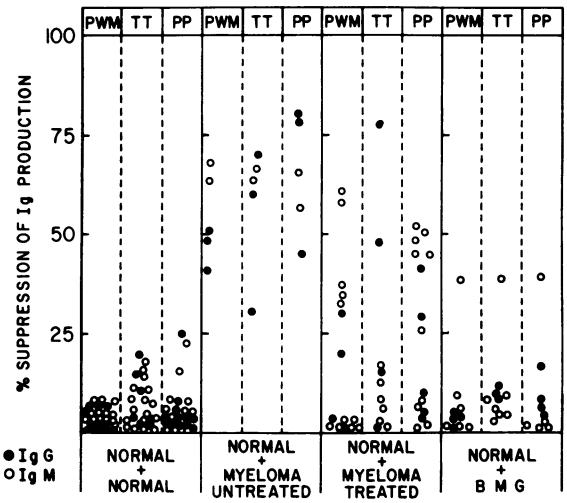

FIGURE 4 Suppression of mitogen- or antigen-stimulated immunoglobulin synthesis of normal PBL by PBL from multiple myeloma patients. Normal PBL (4×10^6 cells) from different normal donors were cultured for 7 days in the presence of PWM, TT, or PP and an equal number of Ficoll-Hypaque-purified monocyte- depleted PBL from 15 allogeneic normal, 5 untreated multiple myeloma, 15 treated multiple myeloma, or 10 BMG patients. The percent suppression of expected immunoglobulin production was calculated as described in Methods.

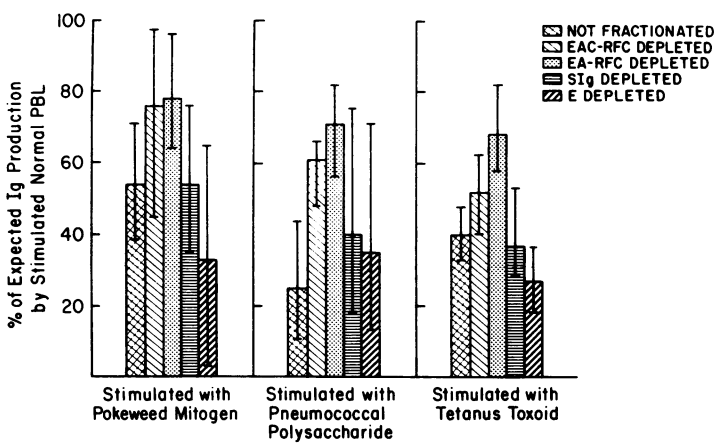

FIGURE 5 Immunoglobulin production by normal PBL co-cultured with myeloma PBL subpopulations. Normal donor PBL (2×10^6 cells) were stimulated for 7 days with PWM (10 μl/culture) in the presence or absence of an equal number of MMC-treated lymphocyte subpopulation-depleted cells from three IgG-untreated and two IgA-untreated multiple myeloma patients. The IgM produced by PWM-stimulated normal PBL (from five different normals) in the presence or absence of IgG multiple myeloma patient cell subpopulations and the IgG produced by PWM-stimulated normal PBL in the presence or absence of IgA multiple myeloma patient cell populations was determined. Percent expected Ig production = (nanograms of Ig produced by PWM-stimulated normal PBL in the presence of myeloma PBL subpopulations/nanograms of Ig produced by PWM-stimulated normal PBL in the absence of myeloma PBL subpopulations) × 100.

present which is independent of a phagocytic monocyte.

Shown in Fig. 5 are the combined results of five separate experiments using lymphocyte subpopulation-depletion techniques to determine which cell populations had suppressor activity. Experiments were done with monocyte-depleted cell populations. Removal of the EA-RFC reduced the observed suppression of in vitro immunoglobulin synthesis more than did the removal of either E-RFL or surface immunoglobulin-bearing cells ($P < 0.01$). In the PWM system, removal of EAC-RFL also reduced the observed suppression more than did the removal of either E-RFL or surface immunoglobulin-bearing cells (*P*

TABLE II

The Effect of Cell Subpopulations from Multiple Myeloma Patients on Suppression of PWM-Stimulated Immunoglobulin Synthesis by Normal Peripheral Blood Lymphocytes

Responder cells stimulated with PWM*	Source of suppressor cell‡	MMC-treated suppressor cell fraction depleted of:	PWM-stimulated immunoglobulin synthesis by normal PBL		
			IgG produced in culture	IgM produced in culture	Suppression
			ng		%
PBLN1			3,896±223		
PBLN1	IgA–untreated myeloma 6 PBL	Unfractionated	148±59		96
		Monocyte depleted	961±192		75
		E-RFL depleted	866±101		78
		EA-RFC depleted	3,050±406		22
		EAC-RFL depleted	2,526±199		35
		SIg depleted	768±89		80
PBLN2				2,800±303	
PBLN2	IgG–untreated myeloma 7 PBL	Unfractionated		588±112	80
		Monocyte depleted		716±99	75
		E-RFL depleted		1,011±206	65
		EA-RFC depleted		2,324±386	20
		EAC-RFL depleted		1,731±207	40
		SIg depleted		809±104	72
PBLN3			3,316±156		
PBLN3	IgA–treated myeloma 12 PBL	Unfractionated	832±50		75
		Monocyte depleted	1,499±202		55
		E-RFL depleted	924±153		72
		EA-RFC depleted	2,809±115		15
		EAC-RFL depleted	2,315±106		30
		SIg depleted	770±95		77
PBLN4				2,599±389	
PBLN4	IgG–treated myeloma 13 PBL	Unfractionated		1,168±183	55
		Monocyte depleted		1,814±203	30
		E-RFL depleted		1,255±301	52
		EA-RFC depleted		2,086±199	20
		EAC-RFL depleted		1,554±323	40
		SIg depleted		1,200±112	54

* Normal donor PBL (1×10^6 cells) from four different patients were mixed with 10 μl/culture PWM in the presence or absence of an equal number of MMC-treated myeloma patient PBL subpopulations.

‡ Myeloma patient PBL were fractionated as described in Methods. Cultures were incubated for 7 days, then the amount of IgG or IgM produced and released into the culture supernates was determined by a double antibody radioimmunoassay. Results = mean nanograms of the appropriate immunoglobulin produced in triplicate PWM-stimulated cultures in the presence or absence of suppressor cells±SE. Percent expected immunoglobulin production = (nanograms of Ig produced in the presence of MMC-treated PBL subpopulations from myeloma patients/nanograms produced in the absence of the MMC-treated suppressor cells) × 100. Percent suppression = 100 − percent expected immunoglobulin production.

TABLE III

The Effect of Depletion of Multiple Myeloma Patient Peripheral Blood Cell Subpopulations on PWM-Stimulated in Vitro Immunoglobulin Synthesis

PWM-stimulated cultures of. . . .	IgG or IgM produced by PWM-stimulated cultures				
	Ficoll-Hypaque purified PBL	Monocyte-depleted PBL	EA-RFC-depleted PBL	E-RFL-depleted PBL	EAC-RFL-depleted PBL
			ng		
Untreated multiple myeloma patients	2,063±443	3,036±333	3,596±452	406±75	3,383±441
	2,414±406	2,616±444	3,816±309	512±44	3,341±485
	1,516±395	1,116±253	2,213±338	411±52	3,031±403
Treated multiple myeloma patients	1,225±406	1,512±298	1,414±212	516±99	1,515±241
	1,611±303	1,689±303	1,518±233	919±122	1,618±222
	1,413±331	1,799±332	1,613±206	716±85	1,616±289
	1,412±401	1,518±317	1,818±219	615±77	1,717±179

Various subpopulation-depleted PBL preparations (2×10^6 cells/culture) were mixed with 10 μl/culture PWM. The amount of IgG or IgM produced after 7 days in culture was calculated. Results represent the mean±SEM. Cultures were done in triplicate.

< 0.05). The EA-RFC is a cell left after carbonyl iron treatment. It is nonphagocytic for latex particles and has no granules staining for nonspecific esterase.

Shown in Table II are experiments involving two untreated multiple myeloma and two treated multiple myeloma patients not included in the studies represented in Fig. 5. Again, in one of the four patients shown in the experiment, phagocytic monocytes appeared to play a significant role in the suppressive effect. In each case studied, removal of EA-RFC and to a lesser extent removal of EAC-RFL diminished the suppressive effect. Removal of T cells (E-RFL) from the cell populations did not decrease the suppressive effect. Additional experiments were done in which normal PBL were mixed with PWM and MMC-treated purified E-RFL, purified EA-RFC, purified monocytes, and purified EAC-RFL from untreated multiple myeloma patients. Suppression of PWM-stimulated IgG or IgM production by normal PBL in coculture was 2–4, 33–37, 13–17, and 32–45% in the presence of untreated multiple myeloma patient peripheral blood E-RFL, EA-RFC, purified monocytes, and EAC-RFL, respectively.

Multiple myeloma patient peripheral blood lymphocyte populations depleted of E-RFL, EA-RFC, EAC-RFL, and monocytes were cultured in the presence of PWM, and the amount of IgG or IgM produced in 7-day cultures was determined. Results are shown in Table III. Three untreated multiple myeloma patients and four treated multiple myeloma patients were tested. In the majority of untreated multiple myeloma patients, PWM-stimulated in vitro immunoglobulin production substantially improved when EA-RFC or EAC-RFL subpopulations were removed. However, removal of any of the above cell populations did not improve PWM-stimulated in vitro immunoglobulin production by PBL from treated multiple myeloma patients.

Suppressor cells for other functions. In previous studies (12), it was shown that PBL from both untreated and treated patients with multiple myeloma had decreased ability to stimulate allogenic normal PBL in a one-way MLC when compared to PBL from normal age-matched patients ($P < 0.05$). Experiments were performed to determine if this defect could be due to suppressor activity. MMC-treated Ficoll-Hypaque-purified PBL from six different untreated patients with multiple myeloma and from five different patients treated for multiple myeloma were each added to one-way MLC between PBL from 10 different allogeneic normal patients. Four out of six of the untreated myeloma patients MMC-treated PBL preparations suppressed the normal allogeneic one-way MLC response greater than 50% (Table IV). In three out of six cases, a supernatant factor could be shown to suppress the normal allogeneic response (results not shown). To determine which lymphocyte subpopulation might be involved in the suppressive effect, lymphocyte subpopulation-depleted PBL from multiple myeloma patients were added to one-way MLC between PBL from four allogeneic normal patients. In three out of the four cases, no suppression was observed when EAC-RFL were depleted, and in one case no suppression was observed when E-RFL were depleted (Table V). No suppressive effects could be demonstrated by PBL from any of the four BMG patients tested, from any of the five treated myeloma patients tested, or from normal PBL tested. It is possible that therapy destroyed the relevant population in the patients who were treated for multiple myeloma. Ficoll-Hypaque-purified preparations were used in these experiments. They were not monocyte depleted before

TABLE IV

The Effect of MMC-Treated PBL from Multiple Myeloma Patients on the in Vitro Response of Normal PBL to Allogeneic Normal PBL in One-Way MLC

Responding cells, normal patient PBL	Stimulating cells, allogeneic normal patient PBL, MMC treated	Suppressor cells, MMC treated	cpm	Inhibition
				%
PBLN1	PBLN1		468±89	
	PBLN2		11,372±552	
	PBLN2	*PBLMM1	8,096±486	+29
	PBLN2	*PBLMM2	3,884±497	+66
	PBLN2	PBLMM8	11,463±501	−1
	PBLN2	PBLMM9	11,616±601	−2
PBLN2	PBLN2		361±59	
	PBLN3		10,216±561	
	PBLN3	*PBLMM3	2,168±152	+79
	PBLN3	*PBLMM4	5,372±189	+47
	PBLN3	PBLMM10	11,163±536	−9
	PBLN3	PBLMM11	10,998±399	−8
PBLN3	PBLN3		353±72	
	PBLN4		9,531±399	
	PBLN4	*PBLMM4	5,258±222	+45
	PBLN4	PBLMM12	10,112±346	−6
	PBLN4	PBLMM8	9,987±499	−5
PBLN4	PBLN4		445±81	
	PBLN5		12,772±1,061	
	PBLN5	*PBLMM5	2,225±206	+83
	PBLN5	PBLMM9	13,063±1,099	−2
	PBLN5	PBLMM10	13,398±699	−5
PBLN5	PBLN5		432±101	
	PBLN6		12,658±766	
	PBLN6	*PBLMM6	2,593±283	+80
	PBLN6	*PBLMM1	8,229±301	+35
PBLN6	PBLN6		399±77	
	PBLN7		10,215±228	
	PBLN7	PBLMM11	10,316±303	−1
	PBLN7	PBLMM12	11,393±406	−11
	PBLN7	*PBLMM5	1,328±317	+87
PBLN7	PBLN7		447±52	
	PBLN8		13,544±1,011	
	PBLN8	*PBLMM1	10,294±256	+24
	PBLN8	PBLBMG1	15,616±846	−15
	PBLN8	PBLMM8	13,868±448	−2
	PBLN8	*PBLMM6	2,398±176	+82
PBLN8	PBLN8		386±23	
	PBLN9		6,952±144	
	PBLN9	*PBLMM2	2,343±337	+66
	PBLN9	PBLBMG2	7,996±246	−15
	PBLN9	PBLMM2	7,073±306	−2
PBLN9	PBLN9		427±44	
	PBLN10		12,212±409	
	PBLN10	*PBLMM3	3,419±222	+72
	PBLN10	PBLBMG3	14,863±252	−22
	PBLN10	PBLMM10	12,998±303	−6
PBLN10	PBLN10		448±99	
	PBLN8		9,993±566	
	PBLN8	*PBLMM4	5,697±442	+43
	PBLN8	PBLBMG4	12,382±604	−24
	PBLN8	PBLMM11	10,199±333	−2

* Indicates that the PBL were from untreated multiple myeloma patients. Normal donor PBL (1×10^5 cells) in 0.5-ml cultures were mixed with an equal number of MMC-treated stimulator cells from allogeneic normal patient PBL in the presence or absence of suppressor cells from multiple myeloma patients or BMG patients. Cultures were incubated for 6 days, then were pulsed for 6 h with 1 μCi/culture [^{125}I]UdR. Results are expressed as mean counts per minute of triplicate cultures±SE. Percent expected counts = (Counts of one-way MLC cultures in the presence of suppressor cells/counts of one-way MLC cultures in the absence of suppressor cells) × 100. Percent inhibition = 100 − percent expected counts. Negative results implied stimulation.

use. This is in contrast to mitogen suppressor cell experiments discussed in the previous section. Suppressive effects were only observed if the suppressor cell to normal cell ratio was 0.5–1 or greater. The greater the suppressor cell to normal cell ratio, the greater the suppression. Controls incorporated into these studies included experiments using three times the amount of [^{125}I]UdR normally added to rule out the possibility that "cold thymidine" might be responsible for the suppressive effect. Results were not significantly different. (Amounts of [^{125}I]UdR in excess of five times the amount normally used were slightly toxic to the cells).

DISCUSSION

The defect in immune function present in patients with multiple myeloma appears to be multifactorial. Our data indicate that in multiple myeloma patients, antigen-binding cells are present in normal frequency, and they are capable of binding antigen. However, the proliferative response to antigen appeared depressed.

Mitogen- or antigen-stimulated production of IgG or IgM in vitro by peripheral blood lymphocytes from patients with multiple myeloma was decreased. PBL from untreated multiple myeloma patients could suppress antigen-stimulated proliferative responses

and immunoglobulin production of normal lymphocytes. Similarly, suppression of normal PBL responses to allogeneic stimuli in one-way MLC in the presence of PBL from untreated myeloma patients could be demonstrated. Suppression of immunoglobulin production in vitro was influenced by the presence of EA-RFC and phagocytic monocytes, whereas, suppression in a one-way MLC was influenced by the presence of EAC and E-RFL.

These results are compatible with the studies of Zolla et al. (2) and Tanapatchaiyapong and Zolla (3). In a mouse model of myeloma, these authors demonstrated a depressed primary immune response, at both B- and T-cell levels which affected the maturation of antibody-forming cells. Broder et al. (5) demonstrated impaired synthesis of polyclonal immunoglobulins in multiple myeloma patients that was mediated by a phagocytic macrophage. In contrast, most patients in our studies demonstrated a significant amount of suppressor activity in the absence of phagocytic macrophages. In our studies, the suppressor activity appeared to be mediated in part by an EA-RFC which was present after carbonyl-iron treatment of blood. This suppression appears to be in addition to that mediated by phagocytic monocytes. Hayward and Greaves (19) have suggested that some cells left behind after carbonyl-iron ingestion have many properties of monocytes but do not phagocytose. They suggest that these cells may correspond to and overlap with a population previously described as null cell. The EA-RFC described in our studies are probably of this type.

The diminished capacity of myeloma patient PBL to stimulate in MLC and their ability to inhibit PBL is similar to the suppressor cell effects reported in Hodgkin's disease (20). The absence of this latter effect in treated myeloma patients suggests that the cells responsible for this effect are sensitive to alkylating agents (12).

Several theories have been proposed to explain the immunological defects in patients with multiple myeloma. First, myeloma cells may release RNA molecules that alter the surface immunoglobulin receptors on B lymphocytes, thus interfering with host recognition of antigen and subsequent antibody formation (21, 22). Second, a feedback inhibition by chalones may block the expansion of B lymphocytes in response to antigen challenge (3, 4). Third, immunoregu-

TABLE V

Suppression of One-Way MLC Reactions Between Normal and Allogeneic Normal PBL by Subpopulations of PBL from Untreated Multiple Myeloma Patients

Responder cells	Stimulator cells	Suppressor cells	Non-fractionated	Suppressor cell depleted populations				
				Monocyte depleted	EAC-RFL depleted	E-RFL depleted	SIg depleted	EA-RFC depleted
PBLN1	PBLN1	—	444±99	—	—	—	—	—
PBLN1	PBLN2	—	11,898±552	—	—	—	—	—
PBLN1	PBLN2	PBLMM2	3,884±208	3,036±388	12,712±999	4,118±289	3,838±289	3,182±449
PBLN1	PBLN2	PBLMM3	2,575±281	4,563±289	10,373±789	2,246±178	2,246±177	2,912±238
PBLN1	PBLN2	PBLMM5	3,323±303	4,991±441	4,820±244	4,586±123	4,352±331	4,821±328
PBLN1	PBLN2	PBLMM6	2,246±344	4,014±228	9,063±443	3,229±176	2,762±289	4,072±299
PBLN2	PBLN2	—	346±89	—	—	—	—	—
PBLN2	PBLN3	—	10,898±662	—	—	—	—	—
PBLN2	PBLN3	PBLMM3	2,586±203	6,696±356	8,616±266	8,063±334	2,767±202	3,036±222
PBLN3	PBLN3	—	358±87	—	—	—	—	—
PBLN3	PBLN4	—	9,439±422	—	—	—	—	—
PBLN3	PBLN4	PBLMM5	2,984±233	5,033±389	3,363±244	5,696±206	3,531±198	3,886±255
PBLN4	PBLN4	—	425±84	—	—	—	—	—
PBLN4	PBLN2	—	12,238±446	—	—	—	—	—
PBLN4	PBLN2	PBLMM6	2,448±289	5,191±443	10,164±777	5,999±289	3,363±288	3,562±263

Normal donor PBL (1×10^5 cells) in 0.5-ml cultures were mixed with an equal number of MMC-treated stimulator cells from allogeneic normal patient PBL in the presence or absence of suppressor cell subpopulations from untreated multiple myeloma patients. Cultures were incubated for 6 days, then were pulsed with 1 uCi/culture [^{125}I]UdR. Results are expressed as mean counts per minute of triplicate cultures±SE.

latory macrophages may inhibit immunoglobulin production (5). Our data demonstrate that there are multiple levels of immune dysfunction in multiple myeloma patients, suggesting that a single theory of pathogenesis is inadequate. In addition, Spitler et al. (23) have presented evidence that decreased levels of C4 and a defect in polymorphonuclear cell adhesiveness in addition to depressed antibody formation may contribute to the development of frequent infections in patients with multiple myeloma.

In the normal immune response, it appears that suppressor mechanisms, both humoral (24, 25) and cellular (26), operate simultaneously to maintain a delicate regulatory balance. There is thus the possibility that the immunoproliferative disease in multiple myeloma is a manifestation of an immune defect in a regulatory compartment and a suppressor function defect may be implicated. In other systems, suppressor functions have not only been attributed to T and B cells (16, 27), but to macropages (28) and nylon-adherent nontheta-bearing cells (29) as well.

Unfortunately, demonstration of suppressive effects in vitro does not dignify their importance in the pathogenesis of the disease in which they are found (30). Suppressor activity has been found after concanavalin A treatment of lymphocytes from normal donors (31). Since plasma cells from multiple myeloma patients have been shown to possess surface antigen capable of eliciting a blastogenic response in autologous PBL (32), and since it has recently been proposed that oncofetal antigen expression of tumors may activate appropriate suppressor cells and thus induce one type of immune tolerance towards a tumor (33), one approach to showing the significance of the observed suppressive effects in multiple myeloma patients would be the demonstration of suppression of lymphocyte blastogenesis to autologous tumor cells by cells added to the mixture of reactor and tumor cells.

Patients diagnosed as having BMG did not show many of the immune defects discussed in this paper. Such patients have in common with myeloma patients the proliferation of plasma cells and the production of excessive amounts of immunoglobulin. The absence of many of immune defects seen in myeloma gives important insight into the pathogenesis of the disease. It is probable that some patients now classified as having benign disease may develop multiple myeloma. Longitudinal studies comparing development of immune defects with the clinical course will be of particular interest.

ACKNOWLEDGMENTS

The authors wish to thank Dr. V. Caggiano and all his patients used in this study for their assistance.

REFERENCES

1. Cone, L., and J. M. Uhr. 1964. Immunological deficiency disorders associated with chronic lymphocytic leukemia and multiple myeloma. *J. Clin. Invest.* **43:** 2241–2248.
2. Zolla, S., D. Naor, and P. Tanapatchaiyapong. 1974. Cellular basis of immunodepression in mice bearing plasmacytomas. *J. Immunol.* **112:** 2068–2076.
3. Tanapatchaiyapong, P. and S. Zolla. 1974. Humoral immunosuppressive substance in mice bearing plasmacytomas. *Science (Wash. D. C.).* **186:** 748–750.
4. Salmon, S. E. 1974. "Paraneoplastic" syndromes associated with monoclonal lymphocyte and plasma cell proliferation. *Ann. N. Y. Acad. Sci.* **230:** 228–239.
5. Broder, S. R., Humphrey, M. Durm, M. Blackman, B. Meade, C. Goldman, W. Strober, and T. Waldmann. 1975. Impaired synthesis of polyclonal (non-paraprctein) immunoglobulins by circulating lymphocytes from patients with multiple myeloma. Role of suppressor cells. *N. Engl. J. Med.* **293:** 887–892.
6. Böyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21** (Suppl. 97): 77–89.
7. Yam, L. T., C. Y. Li, and W. H. Crosby. 1971. Cytochemical identification of monocytes and granulocytes. *Am. J. Clin. Pathol.* **55:** 283–290.
8. Fröland, S. S. 1972. Binding of sheep erythrocytes to human lymphocytes. A probable marker of T lymphocytes. *Scand. J. Immunol.* **1:** 269–280.
9. Greaves, M. F., and G. Brown. 1974. Purification of human T and B lymphocytes. *J. Immunol.* **112:** 420–423.
10. Rabellino, E., S. Colon, H. M. Grey, and E. R. Unanue. 1971. Immunoglobulins on the surface of lymphocytes. I. Distribution and quantitation. *J. Exp. Med.* **133:** 156–167.
11. Froland, S. S., F. Wisloff, and T. E. Michaelsen. 1974. Human lymphocytes with receptors for IgG. A population of cells distinct from Tand Blymphocytes. *Int. Arch. Allergy Appl. Immunol.* **47:** 124–138.
12. Paglieroni, T. G. 1975. Multiple myeloma: an immunological profile in humans. Dissertation, University of California, Davis, Calif. 216 pp.
13. Klinman, N. R. and R. B. Taylor. 1969. General methods for the study of cells and serum during the immune response: the response to dinitrophenyl in mice. *Clin. Exp. Immunol.* **4:** 473–487.
14. Liburd, E. M., and T. A. McPherson. 1973. A simplified technique for the evaluation of antigenbinding cells. *J. Immunol. Methods.* **3:** 79–86.
15. Pellegrino, M. A., S. Ferrone, A. Pellegrino, and R. A. Reisfeld. 1973. A rapid microtechnique for *in vitro* stimulation of human lymphocytes by phytohemagglutinin. *Clin. Immunol. Immunopathol.* **2:** 67–73.
16. Waldmann, T. A., M. Durm, S. Broder, M. Blackman, R. M. Blaese, and W. Strober. 1974. Role of suppressor T cells in pathogenesis of common variable hypogammaglobulinaemia. *Lancet.* **2:** 609–613.
17. Bach, F. H. and N. K. Voynow. 1966. One way stimulation in mixed leukocyte cultures. *Science (Wash. D. C.).* **153:** 545–547.
18. MacKenzie, M. R. and T. Paglieroni. 1977. Multiple myeloma: an immunologic profile. I. Peripheral blood studies. *J. Immunol.* In press.
19. Hayward, A. R., and M. R. Greaves, 1975. Identification of cells with monocyte markers in panhypogammaglobulinaemia. *Scand. J. Immunol.* **4:** 563–570.
20. Twomey, J. J., A. H. Laughter, S. Farrow, and C. C. Douglass. 1975. Hodgkin's disease. An immunodepleting

and immunosuppressive disorder. *J. Clin. Invest.* **56:** 467–475.
21. Giacomoni, D., V. Yakulis, S. R. Wang, A. Cooke, S. Dray, and P. Heller. 1974. In vitro conversion of normal mouse lymphocytes by plasmacytoma RNA to express idiotypic specificities on their surface characteristic of the plasmacytoma immunoglobulin. *Cell. Immunol.* **11:** 389–400.
22. Chen, Y., N. Bhoopalam, V. Yakulis, and P. Heller. 1975. Changes in lymphocyte surface immunoglobulins in myeloma and the effect of an RNAcontaining plasma factor. *Ann. Intern. Med.* **83:** 625–631.
23. Spitler, L. E., P. Spath, L. Petz, N. Cooper, and H. H. Fudenberg. 1975. Phagocytes and C4 in paraproteinaemia. *Br. J. Haematol.* **29:** 279– 292.
24. Uhr, J. W., and G. Möller. 1968. Regulatory effect of antibody on the immune response. *Adv. Immunol.* **8:** 81–127.
25. Hellström, K. E., and I. Hellstrom. 1971. Some aspects of immune defense against cancer. I. In vitro studies on animal tumors. II. In vitro studies on human tumors. *Cancer.* **28:** 1266–1268.
26. Gershon, R. K. 1975. T cell control of antibody production. *In* Contemporary Topics in Immunobiology. N. L. Warner and M. D. Cooper, editors. Plenum Publishing Corporation, New York. **3:** 1–40.
27. Gorczynski, R. M. 1974. Immunity to murine sarcoma virus-induced tumors. II. Suppression of T cell-mediated immunity by cells from progressor animals. *J. Immunol.* **112:** 1826–1838.
28. Kirchner, H., T. M. Chused, R. B. Herberman, H. T. Holden, and D. H. Lavrin. 1974. Evidence of suppressor cell activity in spleens of mice bearing primary tumors induced by Maloney Sarcoma Virus. *J. Exp. Med.* **139:** 1473–1487.
29. Eggers, A. E., and J. R. Wunderlich. 1975. Suppressor cells in tumor-bearing mice capable of nonspecific blocking of in vitro immunization against transplant antigens. *J. Immunol.* **114:** 1554–1556.
30. Siegal, F. P., M. Siegal, and R. A. Good. 1976. Suppression of B-cell differentiation by leukocytes from hypogammaglobulinemic patients. *J. Clin. Invest.* **58:** 109–122.
31. Shou, L., S. A. Schwartz, and R. A. Good. 1976. Suppressor cell activity after Concanavalin A treatment of lymphocytes from normal donors. *J. Exp. Med.* **143:** 1100–1110.
32. MacKenzie, M. R. and T. G. Paglieroni. 1977. Plasma cell antigens in human multiple myeloma. *J. Lab. Clin. Med.* In press.
33. Sinkovics, J. G. 1976. Suppressor cells and human malignant disease. *Br. Med. J.* **2:** 1072–1073.