Comparative Study of the In Vitro Proliferative Responses of Blood and Synovial Fluid Leukocytes of Rheumatoid Arthritis Patients

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ABSTRACT Lymphocyte-rich suspensions from blood and synovial fluid (SF) of 20 patients with rheumatoid arthritis (RA) and from blood of 12 normal subjects, were cultured with heat-aggregated, aggregate-free, and native human gamma globulin (HGG), with autologous IgG separated from RA-SF by anion-exchange chromatography and with phytohemagglutinin (PHA). No significant differences were noted between the in vitro proliferative responses of blood lymphocytes of RA and normal controls to any of these preparations. Significant differences were noted between blood and SF lymphocytes of RA patients with respect to their responses to the aggregate-free HGG and to PHA. Incubation of RA-SF cells but not RA-blood cells with aggregate-free HGG before their culture with the aggregated HGG markedly suppressed the in vitro proliferative response to the latter. The observed differences between blood and SF lymphocytes and the suppression of blastogenic response of SF cells by exposure to the aggregate-free preparation raise the possibility of modulating the immune and/or the inflammatory responses in RA.

INTRODUCTION

The in vitro blastogenic response of human lymphoid cells upon incubation with antigens and phytomitogens is being used extensively to assess the immunocompetence of cells of various lymphoid compartments (1). It has been shown by several investigators that thymus-dependent (TD) [1] cells respond in vitro to phytohemagglutinin (PHA), whereas bone marrow-dependent (BD) cells carry immunoglobulin receptors on their surface (1). Human gamma globulin (HGG) in its aggregated or aggregate-free forms had been shown to be immunogenic or tolerogenic respectively both in vivo (2), and in vitro (3). The association of high levels of rheumatoid factor with more severe disease (4), suggests that antiglobulins may be directly related to, or at least a by-product of the pathogenetic process. One theory of pathogenesis of rheumatoid arthritis (RA) (5) assigns to these antibodies an important role in the production of joint inflammation.

We have investigated the ability of various serum gamma globulin (GG) preparations or synovial fluid (SF) IgG to induce blast transformation of lymphocytes from the blood and synovial fluid of subjects with rheumatoid disease. In this study the following questions were asked: (*a*) Do lymphoid cells within the joint compartment behave in a similar fashion to blood cells with respect to their in vitro interaction with the various GG preparations and PHA? (*b*) Will the exposure of lymphoid cells to the soluble aggregate-free form of GG be capable of blocking their interaction with the aggregate form of GG? In this study we did not intend to compare lymphocytes of RA patients with those from patients with different arthritides but the main purpose was to compare blood with SF lymphocytes of RA patients.

METHODS

The aggregated and aggregate-free GG were prepared from commercial Cohn fraction II of HGG (Miles Labora-

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[1] *Abbreviations used in this paper:* BD, bone marrow-derived; HGG, human gamma globulin; PHA, phytohemagglutinin; RA, rheumatoid arthritis; SF, synovial fluid; TD, thymus-dependent.

tories, Inc., Miles Research Div., Kankakee, Ill.) by heating and centrifugation, as previously described by us (3). After ultracentrifugation, the various globulin preparations were dissolved or suspended in medium 199 with Hanks'-balanced salt solution and $NaHCO_3$ (M-199, Microbiological Associates, Inc., Bethesda, Md.). To avoid the spontaneous formation of aggregates small portions were kept frozen at −20°C; after thawing the solutions were kept at 4°C to avoid possible formation of aggregates by repeated freezing and thawing (6).

The IgG fraction of GG was separated from SF of RA patients by anion-exchange chromatography on columns of diethylaminoethyl cellulose after dialysis against 0.02 M phosphate buffer, pH 6.3, using the same buffer for elution, by slight modification of the method of Restifo, Lussier, Rawson, Rockey, and Hollander (7). The solutions were dialyzed extensively against phosphate-buffered saline solution, pH 7.2, and sterilized by passage through 0.3-μ filters (Millipore Corp., Bedford, Mass.). Radial immunodiffusion against rabbit antihuman IgG, IgM, or whole serum did not demonstrate presence of components other than IgG in the material.

The techniques of blood collection and cell cultures were described by us earlier (8). Briefly, blood was collected in heparin, 100 U/ml blood, and allowed to sediment in sterile plastic tubes (Falcon 2001) placed at 45° angle in an incubator (37°C) for 2 h. The plasma-leukocyte top layer was then collected and washed three times in M-199. The final cell suspension contained 68–82% lymphocytes and was adjusted to contain 10^6 lymphocytes/ml. The cell suspension was then incubated at 37°C for 30 min in serum-free M-199 to allow for shedding (9) of surface-bound proteins. The cells were then washed three times in M-199. 1 ml of the cells suspended in M-199 containing 10% globulin-free pooled AB human plasma were cultured in an atmosphere of 5% CO_2 in air at 37°C as previously described by us (8). Due to the marked tendency of SF cells to clump a different procedure was followed for their separation. The SF collected in heparin, 100 U/ml was immediately mixed with hyaluronidase (Sigma Chemical Co., St. Louis, Mo.), 100 U hyaluronidase/10^7 SF cells. Cells were then incubated for 30 min at 37°C. The fibrin clots were removed by centrifugation at 30 *g* for 5 min. The SF was then centrifuged at 375 *g* for 8 min. By this technique the cells in SF were separated into two layers: top layer containing 47–65% lymphocytes and a bottom layer predominantly made up of neutrophiles and monocytes. The lymphocyte-rich upper layer was then incubated for 30 min in serum-free M-199 at 37°C and then washed three times in M-199 before culturing. The in vitro responses of either blood or SF leukocytes to the various globulin preparations or to PHA (Difco Laboratories, Detroit, Mich.) was assessed by the uptake of [^{3}H]thymidine (8). The optimum duration of cultures were 3 and 5 days for cultures to which PHA or the globulin preparation were added respectively. Cultures designed to study the effects of globulins on the PHA response were kept for 3 days only. The results of all cultures were expressed as specific incorporation which is the ratio of counts per minute (cpm) of incorporated [^{3}H]thymidine of cultures to which mitogens or globulins were added to the cpm of cultures to which no stimulants were added. All cell cultures were done in duplicates. Cell viability was assessed by the trypan blue exclusion method and statistical analysis was done by the Students' *t* test.

20 patients with RA, classical or definite, were studied. Their age ranged between 21–75 yr and had history of arthritis for 1–26 yr. Their latex fixation titer at the time of the study ranged between 0–1280 and they were all on aspirin. Controls were 12 normal healthy volunteers age 25–38 yr.

RESULTS

Table I shows the in vitro proliferative responses of lymphoid cells to the various globulin preparations. It could be seen that though the three types of GG preparations (aggregated, aggregate-free, or native) were more effective in inducing in vitro proliferation of RA blood cells when compared with normal blood cells (Table I, columns 1 and 3), the differences were not statistically significant ($P > 0.01$). Both blood and SF lymphocytes of RA patients did respond to the aggregate HGG. Blood lymphocytes of normals, on the other hand, did not respond to the aggregated GG. Significant lower responses ($P \leqq 0.005$) of RA-SF cells to the aggregate-free preparation were noted when compared with the responses of the RA-blood cells (Table I, line 2, columns 1 and 2). Incubation of RA or normal blood cells with the aggregate-free preparation did not alter to any significant degree their responses to subsequent incubation with the aggregated form (Table I, lines 1 and 4, columns 1 and 3). Significant suppression ($P < 0.01$), on the other hand, was noted upon brief exposure of RA-SF cells to the aggregate-free GG before their incubation with the aggregate form (Table I, lines 1 and 4, column 2). Increased responses of blood cells from normal donors to SF-IgG over the responses to blood cells of RA patients (Table I, line 5, columns 1 and 3) were noted, the differences were not however statistically significant ($P = 0.6$).

Table II shows the proliferative responses of the various lymphoid cells to PHA. It could be seen that in all cases the response of SF cells was significantly different ($P \leq 0.001$) from blood cells. Treatment by hyaluronidase of cell suspensions did not alter the PHA responses of blood cells of either RA or normal controls. Culturing SF cells without prior treatment with hyaluronidase was tried but was unsuccessful due to clumping of cells. The presence of SF-IgG-did not significantly suppress the response of RA cells to PHA (Table II).

DISCUSSION

Previous attempts to demonstrate in vitro transformation of lymphocytes from rheumatoid patients upon incubation with the various globulin preparations have been unsuccessful (10, 11). We felt that further study of this question was needed for several reasons. (*a*) Previous studies used the heat-aggregated globulin or the aggregate-free preparation and did not study the possible modulating effects of the latter on the former. (*b*) Fetal calf serum was used in some studies to support cell growth; such serum might contain nonspecific stimula-

TABLE I

The In Vitro Proliferative Response of Lymphoid Cells from Patients with RA or Normal Controls to Various Globulin Preparations

Additions to cultures (10 μg)	SI* ±SE of lymphocytes from Blood, RA‡ (20)	SF, RA‡ (17)	Blood, Normal‡ (12)
Aggregate HGG	2.76±0.6 (4.1 – 1.7)	2.71±0.2 (3.9 – 1.3)	0.91±0.3 (2.1 – 0.7)
Aggregate-free HGG	2.94±0.4‖ (5.3 – 2.2)	0.61±0.3 (0.9 – 0.5)	1.17±0.4 (2.3 – 0.91)
Native HGG	1.51±0.6 (1.93 – 1.31)	0.69±0.4 (1.1 – 0.52)	0.75±0.2 (1.2 – 0.63)
Aggregate-free HGG, aggregated§	2.92±0.7 (4.7 – 2.1)	0.79±0.3¶ (1.12 – 0.58)	0.89±0.3 (1.31 – 0.71)
SF IgG	1.23±0.3 (2.93 – 0.85)	0.66±0.2 (0.97 – 0.41)	1.93±0.4 (3.71 – 1.01)

* SI, specific incorporation = ratio of counts per min (cpm) of cultures to which various globulins were added to cpm of control cultures to which no globulins were added.

‡ The mean cpm of control cultures of blood, RA were 555 cpm, of SF-RA were 301 cpm and of blood, normal were 736 cpm. The figures are the means, ranges, of the various experiments ± SE. Numbers between brackets at the top of each column are the number of samples tested from the various individuals.

§ Aggregate (agg)-free HGG (10 μg) was incubated with 1×10^6 lymphocytes for 1 h at 37°C, cells were then washed three times and the 10 μg of aggregate HGG was added.

‖ $P < 0.01$ when compared with responses of SF, RA cells to agg-free HGG.

¶ $P < 0.01$ when compared with responses to the agg-HGG of either blood, RA or SF, RA cells.

tory or inhibitory factors (12). (*c*) The amounts of globulin added to the cell cultures were excessive; about 100–1500 μg per 10^6 lymphocytes. The number of antigen receptor sites on an antigen-reactive lymphocyte is approximately 10^5 (14). Even if all 10^6 cells were reactive with globulin, this dose would present each site with approximately 0.7×10^6 molecules per site. A quantity of globulin this large might interfere with the response of the cells, by steric blocking of binding sites or by induction of paralysis in vitro (3). In our experiments we attempted to avoid these possible artifacts by using one pool of globulin-free AB plasma in the culture medium; and by using more "physiologic" doses of antigen, 10 μg, which would provide fewer molecules per cell receptor.

Our results confirm the findings of other investigators (10, 11) who demonstrated that lymphoid cells of blood from RA patients did not differ significantly from those of normal controls with respect to their in vitro responses to the various globulin preparations. Kinsella in a preliminary report (13) was able to demonstrate a higher response of RA blood lymphocytes to the aggregate HGG than that of normal blood lymphocytes. No statistical analysis was given in the report. Though our results with respect to the blood cells were similar, the differences between RA and normal cells were not statistically significant ($P > 0.05$). An interesting new finding was noted in our study. The SF cells were capable of proliferating in vitro in response to the aggregate HGG, but unable to do so upon incubation with the other globulin preparations. Though these cells were viable upon their testing by the trypan blue exclusion method immediately before culturing, we cannot exclude the possibility that their life span in vitro was shorter than that of blood cells. Alternative explanations would be (*a*) SF lymphoid cells and/or synovial lining cells are already engaged in the immune response and their surface Ig receptors are coated with the globulin or the anti-Ig present in the SF and thus are not available for interacting with GG in cell culture (14). This is unlikely since we incubated the cells for 30 min at 37°C before culture. This procedure was shown by others to be effective in shedding off cell-bound proteins and Ig receptors (9). (*c*) Though all cultures were standardized to contain 1×10^6 lymphocytes/culture the number of neutrophils and monocytes in SF cell cultures were three to eight times the number in blood cell cultures and this could have inhibitory effects on the proliferative

TABLE II

The In Vitro Proliferative Responses of Lymphoid Cells from Patients with RA or Normal Controls to PHA

Additions to cultures	Treatment with hyaluronidase*	SI‡ mean, range ± SE of lymphocytes from Blood, RA§	SF, RA§	Blood, Normal§
PHA‖	yes	132±20 (197 − 79) [4]	20±7** (27 − 11) [17]	152±37 (249 − 92) [4]
	no	127±34 (204 − 92) [20]	ND‡‡	105±47 (269 − 58) [12]
PHA + SF, IgG¶	yes	84±23 (149 − 57) [4]	15±9** (31 − 9) [17]	96±14 (127 − 82) [4]
	no	97±29 (172 − 63) [20]	ND‡‡	112±17 (139 − 75) [12]

* Hyaluronidase, 100 U, was added to 10^7 cells for 30 min at 37°C.

‡ SI, specific incorporation = ratio of counts per min (cpm) of cultures to which additions were added to cpm of control cultures to which no additions were added. Means, ranges ± SE are shown.

§ The mean cpm of control cultures of blood, RA were 555 cpm, of SF were 301 cpm, and of blood, normal were 736 cpm. Numbers in brackets are the number of experiments done.

‖ PHA, phytohemagglutinin-M, 0.1 ml of 1:10 dilution added at time 0 to 3 days cultures.

¶ Both PHA and SF IgG (10 μg) are added at time 0 to 3 days culture.

** $P < 0.01$ when compared with responses of either blood, RA or blood, normal.

‡‡ Not done.

potential of SF lymphocytes (12). We do not believe that the latter possibility is an important factor. Preliminary results from our laboratory have demonstrated similar proliferative responses even after excluding neutrophils by means of sucrose plasma linear density gradients. (*c*) The presence of a toxic factor present in the various globulin preparations added to the cultures could account for the poor in vitro responses. This is probably unlikely since the magnitude of the in vitro proliferative responses of the SF cells in response to the aggregate HGG was similar to that of blood cells and the other HGG preparations were not inhibitory to blood cells.

In this study increased responses of both blood and SF cells of RA patients to the aggregate GG were noted. Moreover, SF lymphocytes were incapable of proliferating in vitro in the presence of the aggregate HGG if they were briefly exposed to the aggregate-free HGG and then washed before their interaction with the aggregate GG. These observations could be explained on the basis of increased number of bone marrow-dependent (BD) cells carrying aggregate receptors (15) in these patients. These receptors within the SF compartment probably have higher affinity of binding to the aggregate-free HGG and thus become unresponsive to subsequent exposure to the aggregate HGG. The increase in avidity of SF lymphocyte receptors over those of blood cells could be attributed to the continued compartmental sensitization of SF cells. Mellbye, Messner, DeBord, and Williams (16) have recently shown increased number of B cells carrying Ig receptors in the SF compartment of RA patients when compared with their blood cells. We are planning to extend our in vitro observations to the in vivo system and examine the possibility of modulating the inflammatory response of experimental immune arthritis, induced by immune complexes or aggregate preparations, by means of injections of aggregate-free preparations in the SF compartment.

Our failure to demonstrate significant differences in PHA responses between normal and RA blood lymphocytes would indicate that cell-mediated immunity as expressed by the TD cell function is probably intact in the peripheral blood compartment of RA patients (1). The PHA responses of SF lymphocytes, on the other hand, were poor (Table II). This could not be attributed to the hyaluronidase used to treat the cells since treatment of blood lymphocytes with the enzyme did not affect their PHA responses (Table II). The depressed PHA response could probably be due to few TD cells in the SF. It would therefore appear that in the synovial fluid compartment of patients with RA, there is excess BD cells carrying aggregate GG (Table I) or Ig (16) receptors that are engaged in the effector phase of the immune response (17), and few TD cells responding to PHA (Table II). Similar compartmental imbalance between BD and TD cells had been described in experimental models exhibiting autoimmune phenomena (18) and have been recently postulated by Burnet to be important in the pathogenesis of autoimmunity (19).

Studies designed to understand the immune competence of lymphoid cells in various compartments and at various stages of RA will help in clarifying the possible role that immune mechanisms might play in RA. Attempts at modulating these responses as by the use of tolerogenic antigens as have been tested in this study are encouraged.

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REFERENCES

1. Daguillard, F. 1972. Immunologic significance of in vitro lymphocyte responses. *Med. Clin. N. Am.* **56**: 293.
2. Biro, C. E., and G. Garcia. 1965. The antigenicity of aggregated and aggregate-free human gamma-globulin for rabbits. *Immunology.* **8**: 411.
3. Abdou, N. I., and M. Richter. 1970. The secondary antibody response in tissue culture. IV. Studies of the

in vivo and *in vitro* antigenicity of native, aggregate-free and aggregated human gamma globulin in rabbits. *Immunology.* **18**: 833.
4. Mongan, E. S., R. M. Cass, R. F. Jacox, and J. H. Vaughn. 1969. A study of the relation of seronegative and seropositive rheumatoid arthritis to each other and to necrotizing vasculitis. *Am. J. Med.* **47**: 23.
5. Hollander, J. L., D. J. McCarty, Jr., G. Astorga, and E. Castro-Murillo. 1965. Studies on the pathogenesis of rheumatoid joint inflammation. I. The "R. A. cell" and a working hypothesis. *Ann. Intern. Med.* **62**: 271.
6. Gill, T. J. 1972. The chemistry of antigens and its influence on immunogenicity. *In* Immunogenicity. F. Borek, editor. American Elsevier Publishing Co., Inc., New York. 28.
7. Restifo, R. A., A. J. Lussier, A. J. Rawson, J. H. Rockey, and J. L. Hollander. 1965. Studies on the pathogenesis of rheumatoid joint inflammation. III. The experimental production of arthritis by the intra-articular injection of purified 7S gamma globulin. *Ann. Intern. Med.* **62**: 285.
8. Abdou, N. I. 1971. Immunoglobulin (Ig) receptors on human peripheral leukocytes. II. Class restriction of Ig receptors. *J. Immunol.* **107**: 1637.
9. Uhr, J. W., and E. S. Vitetta. 1973. Synthesis, biochemistry and dynamics of cell surface immunoglobulin on lymphocytes. *Fed. Proc.* **32**: 35.
10. Kacaki, J. N., W. E. Bullock, and J. H. Vaughan. 1969. Failure of lymphocyte transformation in response to gamma-globulins in rheumatoid arthritis. *Lancet.* **1**: 1289.
11. Runge, L. A., and J. A. Mills. 1971. In vivo and in vitro response to autologous IgG in patients with rheumatoid arthritis. *Arthritis Rheum.* **14**: 631.
12. Schellekens, P. T. A., and V. P. Eijsvoogel. 1968. Lymphocyte transformation *in vitro*. I. Tissue culture conditions and quantitative measurements. *Clin. Exp. Immunol.* **3**: 571.
13. Kinsella, T. D. 1972. Enhanced lymphocyte transformation in plasma depleted of complement (C) by aggregated γ-globulin. *Arthritis Rheum.* **15**: 444. (Abstr.)
14. Raff, M. C. 1971. T and B lymphocyte in mice studied by using antisera against surface antigenic markers. *Am. J. Pathol.* **65**: 467.
15. Dickler, H. B., and H. G. Kunkel. 1972. Interaction of aggregated γ-globulin with B lymphocytes. *J. Exp. Med.* **136**: 191.
16. Mellbye, O. J., R. P. Messner, J. R. DeBord, and R. C. Williams. 1972. Immunoglobulin and receptors for C3 on lymphocytes from patients with rheumatoid arthritis. *Arthritis Rheum.* **15**: 371.
17. Abdou, N. I., and M. Richter. 1970. Role of bone marrow in the immune response. *Adv. Immunol.* **12**: 201.
18. Warner, N. L., and M. A. S. Moore. 1971. Defects in hematopoietic differentiation in NZB and NZC mice. *J. Exp. Med.* **134**: 313.
19. Burnet, M. 1972. Modern immunological theory. *In* Autoimmunity and autoimmune disease. M. Burnet, editor. F. A. Davis Company, Philadelphia. 33.