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Induction of Autoantibody-Producing Cells after the Coculture of Haptenated and Normal Human Mononuclear Leukocytes (41247)[1]

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Abstract. The coculture of normal human peripheral blood mononuclear leukocytes (PBL) and autologous mononuclear leukocytes coupled to the trinitrophenyl (TNP) hapten (TNP-PBL) was found to induce a polyclonal activation of antibody-producing cells. The polyclonal activation of antibody-producing cells was demonstrated by detecting the induction of cells producing antibody to sheep red blood cells using a complement-dependent, direct, hemolytic plaque-forming cell (PFC) assay. A ratio of four normal to one haptenated mononuclear leukocyte was found to be optimal for inducing the polyclonal activation of antibody-producing cells in these cultures. The plaque-forming cell assay in these experiments utilized monolayers of indicator red cells. Further evidence for the polyclonal induction of antibody-producing cells by TNP-PBL was provided by demonstrating PFC on monolayers of not only sheep red blood cells, but also autologous human red cells, bromelain-treated autologous red cells, TNP-coupled human and sheep red cells, and human autologous red cells coupled to human heat-aggregated IgG with chromic chloride. Thus cells secreting antibody to TNP, human red cells,and human IgG were induced. Anti-IgG and anti-human red cell-producing cells were first detected on Day 2 of culture and were still present on Day 9. Mononuclear leukocytes altered by chemical haptenation polyclonally stimulate normal mononuclear leukocytes to become antibody-producing cells. This polyclonal stimulation of antibody-producing cells includes cells producing antibodies to human IgG and human autologous red blood cells suggesting that autoantibody-producing cells are induced.

In both the autoimmune disease systemic lupus erythematosus (1) and in the animal model for this disease the NZB/NZW mouse (2) there is a polyclonal induction of antibody-producing cells. The stimulus for this polyclonal induction of antibody-producing cells is unknown, but could be similar to the stimuli that induce cytotoxic lymphocytes. Cytotoxic lymphocytes are induced by cells with new cell surface antigens or altered cell surface antigens. New cell surface antigens have been described on the surface of lymphocytes from patients with systemic lupus erythematosus (3). The new cell surface antigens that induce cytotoxic lymphocytes may be viral (4) or tumor associated (5). Chemically haptenated lymphocytes, a model for altered self, also induce cytotoxic lymphocytes (6–8). It has been demonstrated that the coculture of autologous haptenated lymphocytes and normal lymphocytes leads to blast transformation as measured by the uptake of tritiated thymidine (9). In the present studies, haptenated mononuclear leukocytes were found to induce normal mononuclear leukocytes to produce antibodies polyclonally. As part of this response, autoantibody-producing cells are induced.

Materials and Methods. *Mononuclear leukocytes.* Peripheral blood mononuclear leukocytes (PBL) were obtained by venipuncture on healthy, young-adult volunteers. Heparinized blood (80–200 ml) was obtained and cells were isolated on a Ficoll–Hypaque gradient (10). PBL were

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washed twice in Hepes-buffered Hanks' balanced salt solution (HBSS; Gibco, Grand Island, N.Y.) pH 7.8. Wright stain morphology revealed the cells to be 90% lymphocytes and 10% monocytes. Cells were ≥98% viable by trypan blue dye exclusion after separation and washing.

Trinitrophenyl (TNP) modification of PBL. Washed PBL were coupled to 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) (Sigma, St. Louis, Mo.) using Seldin and Rich's (9) modification of Shearer's technique (6). Briefly, PBL were incubated at 37° for 12 min in 2 m*M* TNBS in Hepes-buffered HBSS, pH 7.8. Unreacted TNBS was removed by washing the cells three times in Hepes-buffered HBSS, pH 7.8, supplemented with 5% heat-inactivated (30 min, 56°) fetal bovine serum (GIBCO), and 10 mg/ml glycylglycine (Sigma). The yield after TNP coupling was approximately 50%, but after washing cell viability was ≥98%. Cells treated with mitomycin (Sigma) were incubated with a 50 μg/ml solution for 30 min.

Cell culture techniques. Normal PBL (5×10^6) were cocultured with varying numbers of TNP-coupled PBL in a 5% CO_2 humidified atmosphere at 37° in 17 × 100 polystyrene culture tubes (Falcon 2001, Cockeysville, Md.). Cell media consisted of 10 ml of RPMI 1640 (Flow Laboratories, Rockville, Md.) supplemented with 1% antibiotic–antimycotic (Gibco), 2 m*M* L-glutamine (Gibco), and 10% pooled, heat-inactivated (30 min, 56°) human AB serum (Gibco). Prior to culture, the human AB serum was absorbed (0°, 60 min) with the appropriate red cell (human or sheep) to be utilized in the plaque assay. Cultures were fed with 1 ml of supplemented media on Days 2 and 5, and on Day 8 the cultures were spun down and fresh supplemented media was added.

Plaque-forming cell (PFC) assay. PFC were assayed on Day 7 unless otherwise indicated using a modification of the method of Dosch and Gelfand (11, 12). Red cell monolayers were coupled to flat-bottom, 96-well, cluster plates (Dynatech Laboratories, Alexandria, Va.) with poly-L-lysine (25 μg/ml) (Sigma). Plates containing 25 μl of poly-L-lysine per well were incubated at 37° for 30 min and washed three times with phosphate-buffered saline (PBS), pH 7.4. One hundred microliters of a 5% red cell suspension (100 μl) was added to each well and plates were spun at 1000*g* for 5 min and then incubated at 37° for 1 hr. Unbound red cells were washed off with PBS and monolayers were overlayed with 75 μl of RPMI 1640 until use. Monolayers were then inspected with an inverted microscope and only intact monolayers were utilized.

Sheep red blood cells (SRBC) used for preparing indicator monolayers were drawn from stock animals directly into Alsever's solution and stored at 4°. Human autologous red cells (HRC) were obtained at the time of PBL separation and stored as a 50% solution in RPMI 1640 at 4° until use.

HRC were either used without alteration, treated with bromelain, or coupled to heat-aggregated IgG or TNP. Bromelain treatment was carried out using the method of Cunningham (13). Briefly, HRC were incubated at a bromelain (Sigma) concentration of 10 mg/ml in PBS and washed three times in PBS before use. Heat-aggregated IgG was obtained by dissolving human pooled Cohn Fraction II IgG (U.S. Biochemical, Cleveland, Ohio) in normal saline at a concentration of 10 mg/ml and heating this solution at 63° for 20 min. Insoluble aggregates were removed by centrifugation at 10,000*g* for 10 min. Heat-aggregated IgG was coupled to HRC with chromic chloride using the method of Sweet and Welborn (14). Heat-aggregated IgG was used at a concentration of 5 mg/ml. After protein coupling, the HRC were suspended in 0.1% gelatin in normal saline until use. HRC and SRBC were coupled to TNP using the method of Rittenberg (15).

Cultures were utilized if there was ≥90% viability before the assay. Cultured PBL (5×10^3 to 10^5 cells) were added to each well after two washings with RPMI 1640. Guinea pig serum was used as a source of complement and was obtained by cardiac puncture and stored at −70°. This serum was absorbed with the appropriate indicator red cell at 0° for 60 min, diluted 1:20 or 1:30 in normal saline, and 25 μl of this solution was

added to each well. Plates were then incubated at 37° for 1 hr. Plaques were then counted with an inverted microscope. At least five wells were counted per culture.

Results. *Optimal ratio of normal PBL to TNP-PBL for inducing anti-SRBC PFC.* In the first series of experiments shown in Fig. 1, 5×10^6 normal PBL were cultured with varying numbers of TNP-PBL in order to determine the optimal ratio of normal PBL to TNP-PBL that would cause a polyclonal induction of PFC as evidenced by the induction of anti-SRBC PFC. Monolayers of SRBC were used as the indicator cell. Anti-SRBC PFC were induced at all concentrations of TNP-PBL, but the largest number were found at the ratio of 4:1, normal PBL:TNP-PBL. Control cultures, containing only normal PBL, contained small numbers of anti-SRBC PFC. The optimal 4:1 ratio was utilized in subsequent experiments.

Determination of anti-TNP PFC using TNP-SRBC as the indicator cell. In these experiments normal PBL and TNP-PBL were cocultured at the 4:1 ratio that optimally stimulated anti-SRBC PFC. Cultures were assayed on SRBC and TNP-SRBC indicator monolayers and the results are shown in Fig. 2. The difference between the number of PFC on the two monolayers indicates that anti-TNP PFC response was induced. Control cultures, containing no TNP-PBL, rarely contained PFC.

Induction of PFC to autologous HRC. In these experiments the same 4:1 ratio of normal PBL to TNP-PBL was utilized to induce PFC. As seen in Fig. 3, when untreated autologous HRC were used as the indicator cell monolayer, anti-HRC PFC were identified in the cultures containing TNP-PBL. Thus, cells were induced that produced antibody to autologous red cells.

Induction of PFC to bromelain-treated autologous HRC. In these experiments also shown in Fig. 3, autologous HRC were pretreated with bromelain and monolayers were formed. Cultures consisted of the same 4:1 ratio of normal PBL to TNP-PBL. Large numbers of PFC were induced in cultures containing TNP-PBL and there

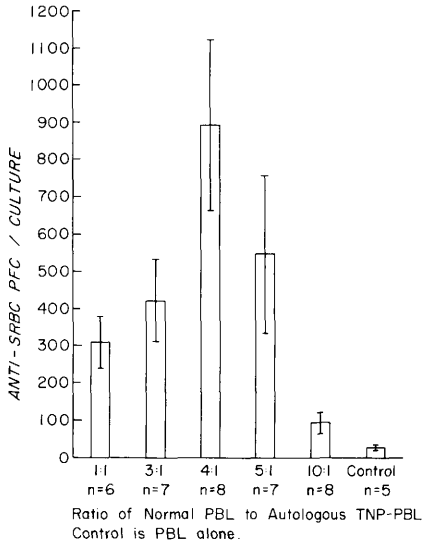

FIG. 1. Optimal ratio of normal PBL to TNP-PBL for the induction of anti-SRBC PFC. Normal PBL (5×10^6) were cocultured with varying numbers of TNP-PBL and assayed on Day 7 for PFC on indicator cell monolayers of SRBC. Control cultures contained 5×10^6 PBL alone. The data are shown as the mean ± SEM. n is the number of cultures. Five individuals were utilized for each determination.

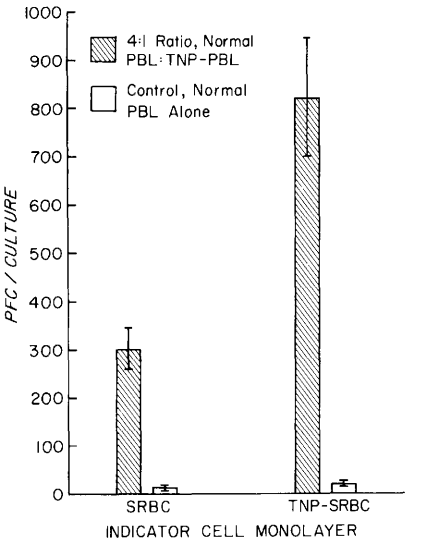

FIG. 2. Induction of anti-TNP PFC. Normal PBL (5×10^6) were cocultured with 1.25×10^6 TNP-PBL and assayed on Day 7 on indicator cell monolayers of TNP-SRBC and untreated SRBC. Control cultures contained 5×10^6 PBL alone. The data are shown as the mean ± SEM of four experiments consisting of triplicate cultures. Four individuals were utilized.

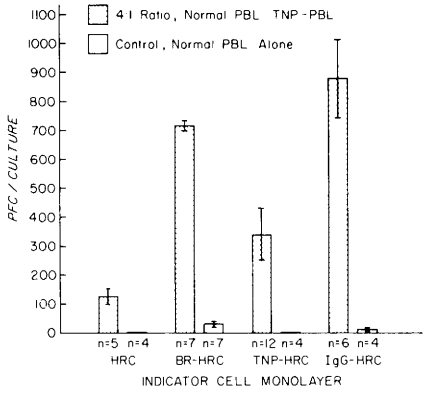

FIG. 3. Demonstration of the induction of PFC to autologous red cells, IgG, and TNP using autologous HRC in the indicator cell monolayer. Normal (5×10^6) PBL were cocultured with 1.25×10^6 TNP-PBL and assayed on Day 7 on indicator cell monolayers consisting of untreated autologous HRC alone or autologous HRC treated with bromelain. In addition, untreated autologous HRC were coupled to TNP or coupled to aggregated human IgG with chromic chloride. Control cultures consisted of 5×10^6 PBL alone. The data are shown as the mean ± SEM. n is the number of cultures. At least two individuals were utilized for each determination.

was only a minimal response in control (PBL alone) cultures. In addition, the number of PFC on bromelain-treated autologous HRC was much greater than the number on untreated autologous HRC, suggesting that new antigenic determinants had been exposed on the HRC surface during bromelain treatment.

Induction of anti-IgG (rheumatoid factor) PFC. In these experiments also shown in Fig. 3, the 4:1 ratio of normal PBL to TNP-PBL induced a higher number of anti-IgG PFC when compared to control cultures containing normal PBL alone. The indicator monolayer consisted of autologous HRC coupled to human heat-aggregated IgG with chromic chloride. The response in cultures containing TNP-PBL was much greater on these monolayers than on autologous untreated HRC monolayers (Fig. 3), thus PFC making antibody to human IgG (rheumatoid factor) were induced.

Determination of anti-TNP PFC using TNP-HRC as the indicator cell. In these experiments (Fig. 3) we demonstrate an anti-TNP response using TNP-coupled autologous HRC as the indicator cell in the cultures containing normal PBL to TNP-PBL at a 4:1 ratio. Control cultures, containing normal PBL alone, did not contain appreciable PFC on the TNP-HRC monolayers. The number of PFC on TNP-HRC was much greater than the number of PFC on untreated autologous HRC monolayers alone, thus there was induction of anti-TNP PFC as was also demonstrated using TNP-SRBC monolayers (Fig. 2).

Comparison of irradiation, mitomycin treatment, or TNP coupling alone on the stimulatory activity of TNP-PBL. In order to determine if the proliferation of TNP-PBL was responsible for the induction of PFC, proliferation by TNP-PBL was inhibited by irradiation or mitomycin treatment. Simultaneous cultures were prepared using untreated TNP-PBL, irradiated TNP-PBL (2000 rad), and mitomycin-treated (50 μg/ml) TNP-PBL. The optimal 4:1 ratio of normal PBL:TNP-PBL was utilized and PFC were enumerated on HRC and HRC-IgG monolayers. As seen in Fig. 4, there was no significant difference between

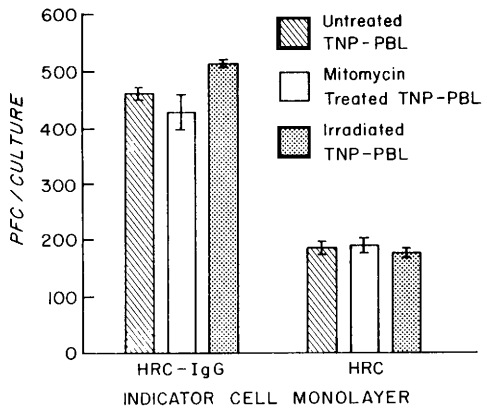

FIG. 4. Effect on the autoantibody PFC response when TNP-PBL were either untreated or treated to inhibit proliferation. Normal PBL (5×10^6) were cocultured with 1.25×10^6 TNP-PBL either untreated, mitomycin-treated at the start of cultures (50 μg/ml), or irradiated at the start of cultures (2000 rad). Cultures were harvested on Day 7 and PFC were enumerated on either autologous HRC or autologous HRC coupled to aggregated IgG with chromic chloride. The data are shown as the mean ± SEM of three experiments containing triplicate cultures. Three separate individuals were utilized.

the number of anti-HRC PFC or anti-IgG PFC induced by treated or untreated TNP-PBL. This suggests that the proliferation of TNP-PBL is not responsible for the stimulatory activity seen in these experiments.

Time course of the PFC response. In these experiments, the anti-IgG PFC and anti-HRC PFC response was evaluated over 9 days. The optimal 4:1 ratio of normal PBL:TNP-PBL was utilized. As seen in Fig. 5, PFC were not detected on Day 1, but rose rapidly on Day 2, and the response was not significantly different on subsequent days. The response on Days 8 and 9 were highly variable, with some individuals having a dramatic increase in numbers of PFC and others having a decrease in the numbers of PFC. Monolayers of HRC and IgG-HRC were used, thus the difference between the two values represented an anti-IgG PFC (rheumatoid factor PFC) response.

Discussion. In these experiments, we have demonstrated that in an autologous human *in vitro* system, antibody-producing cells as measured by a PFC assay can be induced after haptenated PBL are cocultured with normal PBL. The antibody-producing cells are induced polyclonally as evidenced by the induction of a high number of anti-SRBC PFC.

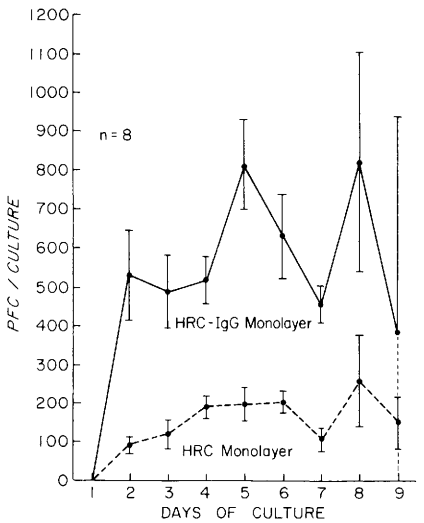

FIG. 5. Time course in days of the autoantibody PFC response. Normal PBL (5×10^6) were cultured with 1.25×10^6 TNP-PBL and assayed on monolayers of either autologous HRC or autologous HRC coupled to aggregated IgG with chromic chloride. The data are shown as the mean ± SEM of eight separate cultures utilizing five individuals.

In systemic lupus erythematosus, the prototype of human autoimmune disease, there is a polyclonal induction of PFC, both in humans with the disease (1) and in the animal model for this disease the NZB/NZW mouse (2). The mechanism for this response is not completely understood and is likely to be multifactoral. Experimental evidence of decreased T-lymphocyte suppressor function in the human disease (16) and in the NZB/NZW mouse (17) may be one important factor, but increases in activity of helper factors produced by T lymphocytes may also be important. Both antigen-specific helper factors (18) and nonspecific helper factors that induce antibody-producing cells polyclonally (19–21) have been obtained from antigen-stimulated mononuclear cultures. Recently helper factors that stimulate human B lymphocytes polyclonally have been found in long-term cultures of human lymphocytes that were previously sensitized to haptenated lymphocytes and then rechallenged with these cells (22). The polyclonal induction of antibody-producing cells in the present studies may also be due to the release of a helper factor.

In these experiments, we have demonstrated the induction of autoantibody-producing cells as part of the polyclonal response. Anti-IgG (rheumatoid factor)-producing cells were induced and detected using a PFC assay in which the indicator cell was an autologous red cell coupled to heat-aggregated IgG with chromic chloride. The agglutination of human red blood cells coupled to heat-aggregated IgG with chromic chloride has recently been shown to be an excellent method of demonstrating anti-IgG (rheumatoid factor) activity in the serum of patients with rheumatoid arthritis (23). The rheumatoid factor PFC detected in the present studies were direct plaques and were therefore of the IgM class as are most human rheumatoid factors. In a murine system, Dresser (24) used lipopolysaccharide to induce a large number of direct anti-IgG (rheumatoid factor) PFC as part of a polyclonal response.

In this study, cells producing antibody to autologous red cells were also induced. Anti-red cell antibodies are a prominent finding in animal models of autoimmunity (25) and they frequently occur in human systemic lupus erythematosus. We were able to demonstrate increased numbers of cells producing antibody to autologous red cells when the red cells were treated with bromelain, which exposes new antigenic determinants on the surface of red cells (13, 26).

The induction of cells producing antibodies to IgG, TNP, human red cells, and sheep red cells reproduces one of the few persistent features of animal and human autoimmune disease, a polyclonal activation of antibody-producing cells. If mononuclear leukocytes altered by haptenation can induce this response, then it is possible that virus transformed cells may also do this.

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