Immunologic Function of Antigen-Induced Peripheral Blood Lymphoblastoid Cells[1] (35504)

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In short-term cell culture of peripheral leukocytes, the antigen-specific lymphocyte transformation response has been shown to parallel the *in vivo* formation of the immunoblast during either a cellular or humoral immune response (1–3). Committed small lymphocytes react with the appropriate sensitizing antigen and by processes of differentiation and/or proliferation produce clones of cells responsible either directly or indirectly for synthesizing products capable of mediating that particular immune response (1, 4, 5). These include specific immunoglobulins and a number of factors demonstrated recently in cultures of cells from animals exhibiting delayed hypersensitivity following stimulation with the specific antigen. The latter products include migration inhibitory factor (6), lymphotoxic factor (7, 8), chemotactic factor (9), and mitogenic factor (10, 11).

The *in vitro* biologic phenomenon, which is consistently observed and which may play a role in the synthesis of all of the above products, is the blast transformed lymphocyte. Previous work in this laboratory (12) has shown that the process of lymphocyte transformation *in vitro* can be observed in peripheral lymphocytes from rabbits immunized to favor either humoral antibody production or a state of delayed hypersensitivity. It seemed worthwhile therefore, to determine what immunologic products were elaborated *in vitro* following stimulation of cell cultures from these animals. The results of these studies are presented below.

Materials and Methods. Normal, male New Zealand rabbits, initially weighing 8–10 lb were immunized with washed chicken erythrocytes (C-RBC) suspended in either saline and injected intravenously, or in Freund's complete adjuvant and injected subcutaneously. The dosages and regimens of injection used to induce either a marked humoral antibody response without evidence of delayed hypersensitivity, or to favor the latter response without significant antibody formation have been described (12). Serum taken from the animals was assayed for antibody by a direct hemagglutination titration. The animals were also skin tested to detect a delayed hypersensitivity response using as the antigen a urea extract of C-RBC prepared according to the method described by Boyden (13).

In vitro *transformation.* The degree of lymphocyte stimulation in cultures maintained for 4 days was determined by direct observation of stained smears for percentage of lymphoblastoid forms, and by measurement of the incorporation of tritium-labeled nucleic acid precursors. The culture system was essentially that described by Sell and Gell (14). Blood drawn at various times from the central ear artery was defibrinated, sedimented by the addition of pig skin gelatin (Wilson Company, Chicago, Illinois), and the leukocyte-rich supernatant was removed and placed in cultures as previously described (12). Some cultures were stimulated by addition of the C-RBC to the system while others were maintained without the addition of specific antigen, as unstimulated controls.

In vitro *immunoglobulin formation.* Specific antibody to determinants on the C-RBC was detected by a modification of the plaque technique of Jerne *et al.* (15). Both the direct micromethod, described by Mosier and Coppelson (16); and the indirect assay, using essentially the methods described by

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Sterzl and Riha (17), were used. Both 19S and 7S antibody could be detected under these conditions. The lymphocyte cultures were maintained for 96 hr and were terminated with a 4-hr pulse of tritiated thymidine (^{3}HT) (NET 027 X, 15 Ci/mmole) (New England Nuclear, Boston, Mass.), as previously described (12). Nonpulsed cultures were also prepared to determine background counts in subsequent procedures. The cells were washed several times with saline to remove exogenous label and were dispersed by gentle mixing for 15 to 30 sec to produce a suspension of single cells. The cell suspension was added to a mixture of 0.4 ml of 0.5% agarose (L'Industrie Biologique Francaise S.A., Gennevilliers, France) in Eagle's minimal essential medium and 0.05 ml of 4% C-RBC at 42°. The suspension was again gently mixed and poured quickly onto a microscope slide that had been previously coated with a 0.1% agarose solution to eliminate toxicity of the glass surface for the cells. After the cell mixture on the slides had been allowed to gel, 1.0 ml of 10% solution of guinea pig serum (fresh frozen at —70°) as a source of complement, was added to flood each slide. The slides were placed in a humidified 5% CO_2 incubator at 37° for 2 hr and then stored for 18 hr in an airtight container at 4° to prevent evaporation. The resulting hemolytic plaques were counted using a dissecting microscope. The data are expressed as the number of plaque-forming cells (PFC)/3 $\times$ 10^6 cells placed into culture. The procedure for the indirect method was carried out in the same manner except that 0.05 ml of a 1:3.5 dilution of adsorbed, inactivated, goat antirabbit 7S antibody (Immunology Inc., Glen Ellyn, Ill.) was added to the agarose.

Autoradiography. The same slides prepared for the detection of PFC as described above were used for the autoradiographic observations. Complement was allowed to drain from the slides and they were rinsed with normal rabbit serum (1:6 dilution in H_2O), and again allowed to drain, according to the technique of Berglund (18). The slides were dried in cool air from a fan for 1 hr and finally were fixed in methanol for 30 min. After drying, the slides were sprayed with Omnispray (Isolab Inc., Elkhart, Ind.), warmed to 37°, and dipped into warmed Kodak NTB2 Nuclear Track Emulsion (Standard Photo, Chicago, Ill.). The coated slides were placed in a slide box and exposed at —70° for 48 hr. They were then processed with Dektol developer and Acid Fix (Kodak) with distilled water rinses according to the instructions provided by the manufacturer (19). The unstained preparations were observed for labeled cells at the center of plaques with a phase contrast microscope at a magnification of 510$\times$.

Results and Discussion. Our initial experiments were designed to examine the possible role of the transformed lymphocyte *in vitro* in terms of specific immunoglobulin production. It was felt that the antigenic stimulation of peripheral blood cells in culture might induce the small lymphocytes from hyperimmunized animals to proliferate into antibody producing cells thus showing an anamnestic response *in vitro*. Cells from animals exhibiting either marked delayed hypersensitivity without detectable antibody, or a hyperimmune antibody response without evidence of positive delayed skin reactions, after 4 days in culture were found to be stimulated by specific antigen as determined by morphologic criteria and thymidine uptake. Table I shows the results of assays of these cultures for hemoltyic plaque-forming cells. In spite of the period of *in vitro* culture prior to the plaquing procedure, and the manipulations during this procedure itself, which might reduce the survival or activity of stimulated cells, significant numbers of plaques were observed in the hyperimmune antibody-producing animal cell cultures. It is of interest to note that the antibody found was primarily of the IgG class, which tends to predominate in the anamnestic response. The presence of such antibody-forming cells in cultures from normal animals or animals exhibiting delayed sensitivity to the antigen could not be demonstrated under the conditions of these experiments. The IgG type response following *in vitro* stimulation suggests that previously committed small lymphocytes, found in the peripheral circulation, can participate in an anamnestic humoral response.

TABLE I. Plaque-forming Cells from *in Vitro* Lymphocyte Cultures of Peripheral Blood from Hyperimmunized Rabbits.

Blood taken from animals immunized to favor:	*In vitro* stimulant	Plaques/3 × 10⁶ cells placed in culture	
		Direct method (19S)	Indirect method (19S + 7S)
Delayed hypersensitivity	None	1.00 ± 1.00[a]	6.00 ± 1.73
	C-RBC	1.67 ± 2.08	2.67 ± 1.53
Antibody formation	None	6.33 ± 2.52	13.00 ± 2.00
	C-RBC	4.67 ± 5.03	27.33 ± 3.78
Neither (normal)	None	3.33 ± 1.15	3.00 ± 1.00
	C-RBC	6.33 ± 1.53	3.00 ± 1.00

[a] Each value represents the mean of three cultures ± standard deviation. Statistical analysis by the t test showed significant differences in the following samples from the indirect plaque assay: normal unstimulated: antibody unstimulated, $p < 0.005$; normal stimulated: antibody stimulated, $p < 0.001$; delayed unstimulated: antibody unstimulated, $p < 0.025$; delayed stimulated: antibody stimulated, $p < 0.005$; antibody unstimulated: antibody stimulated, $p < 0.010$.

To determine whether or not the blast cells formed during lymphocyte stimulation *in vitro* could be directly implicated with the antibody production observed, positive identification of the cells at the center of the plaques was attempted using autoradiography. In a series of 5 separate experiments using peripheral blood from 3 animals hyperimmunized intravenously to favor an antibody response, labeled blastoid cells were found at the center of some hemolytic plaques, although labeled cells were also found in other areas of the preparations, not associated with plaques. The number observed in different experiments ranged from 1 to 8 per 1000 blast cells counted, with a mean of 3 per 1000. This suggests that some, but not all blast cells in the cultures were producing specific hemolysins to C-RBC at the time tested. It should be appreciated that these numbers represent minimal values, since these determinations were made on cells that had been in culture for 4 days. Cell death during this period of culture as well as the manipulations involved in plaqueing and subsequent autoradiography of these preparations would tend to decrease the chances of demonstrating specific antibody-forming blast cells under the conditions described. Figure 1 shows one of the autoradiographically labeled blast cells at the center of a hemolytic plaque.

Direct demonstration of antibody production by blastoid cells induced *in vitro* in a system such as that described has not previously been reported to our knowledge. Although the presence of immunoglobulin in cultured cells and in the culture medium has been observed (20, 21), an immunologic specificity of these globulins has been difficult to establish. Girard (22) has demonstrated specific antibody to penicillin in culture supernatants of *in vitro* stimulated lymphocytes from patients with penicillin hypersensitivity. Lamvik (23) has shown a correlation between blast transformation and the development of plaques as determined by the Cunningham assay (24) in cell cultures from

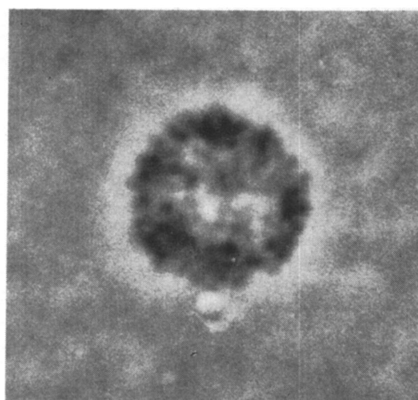

FIG. 1. Autoradiograph of a tritium-labeled lymphoblast at the center of plaque in a lawn of C-RBC; noncritical focus results from the nature of the preparation; ×7040.

rabbits hyperimmunized with sheep erythrocytes. By using a plaqueing technique in combination with autoradiographic identification of the plaque-forming cell, the studies reported here provide additional evidence to implicate at least a small subpopulation of peripheral lymphocytes with *de novo* synthesis of specific antibody.

While no techniques are currently available to identify a single cell as being responsible for the synthesis of one of the factors correlated in recent years with delayed type hypersensitivity, it seemed appropriate to demonstrate at least some of these factors in cell cultures from animals appropriately immunized and used in the studies described above. Accordingly, the skin sensitizing factor described by Bennett and Bloom (25) was demonstrated in dialyzed culture supernatants from appropriately stimulated animals. That is, supernatants, dialyzed and concentrated 10 times, from cell cultures prepared from blood drawn from animals exhibiting a marked delayed hypersensitivity to C-RBC and maintained for 4 days in the *absence* of the antigen, when injected into the skin of normal guinea pigs in 0.1-ml volume resulted in induration with a mean diameter of <5 mm. Identical preparations from cells cultured in the *presence* of C-RBC induced positive skin reactions ranging from 20 to 22.5-mm induration. Similarly *in vitro* stimulated cell cultures from hyperimmune antibody producing animals produced supernatants, which, when concentrated and injected as above, gave skin reactions ranging from 8 to 12-mm induration. These animals had received intravenous booster injections of washed C-RBC at irregular intervals during the course of these experiments to maintain them in a state of maximum antibody prduction. The slight skin sensitizing reactions elicited by supernatants from their cultured cells, suggests that at the time of testing they were also showing a low level of delayed hypersensivity.

It was also possible to demonstrate migration inhibitory factor (MIF) with cell culture supernatants prepared as described above. These experiments were carried out essentially as described by David (26), except that normal CF 1 mouse peritoneal macrophages, cultured in medium 109 supplemented with 25% horse serum, were used in the migration chambers. Supernatants from normal, antibody-producing, and delayed sensitive animal lymphocytes cultured with or without C-RBC stimulation were dialyzed, lyophilized, and reconstituted in medium 109. Results of experiments to detect MIF in each of these preparations are shown in Table II. The factor could be detected in cell cultures from both antibody-producing and delayed sensitive animals following *in vitro* stimulation. The delayed hypersensitive animal sensitivities were waning at the time these experiments were done. They had been sensitized approximately 1 year earlier. This loss of sensitization was reflected in decreased thymidine incorporation in cultures done at this time. The finding of activity in the antibody-forming animal cell cultures again suggest the background activity seen in the skin sensitizing experiments above.

TABLE II. Production of Migration Inhibitory Factor by Peripheral Lymphocytes from Hyperimmunized Rabbits Cultured *in Vitro*.

Blood taken from animals immunized to favor:[a]	*In vitro* stimulant	Migration (%) compared to:	
		Controls[b]	Unstimulated
Delayed hypersensitivity	None	112.6	—
	C-RBC	66.4	59.0
Antibody formation	None	78.4	—
	C-RBC	36.0	46.0
Neither (normal)	None	95.6	—
	C-RBC	80.3	83.8

[a] Supernatants from 4-day cultures were dialyzed, lyophilized, and redissolved in medium 109, containing 25% horse serum. Supernatants from normal cells concentrated 2×; all other supernatants, concentrated 3×.

[b] Migration of macrophages from capillaries cultured in medium 109 containing 25% horse serum.

An additional factor associated with blast transformation *in vitro*, and correlated with delayed-type hypersensitivity, is the release of lymphotoxin (LT). This substance can be detected indirectly by determining the incorporation of ^{14}C-labeled amino acids into

HeLa cell monolayers in the presence or absence of LT. Decreased incorporation is a reflection of cell destruction. Using essentially the methods described by Granger and Kolb (27), supernatants from delayed sensitive animals cell cultures, stimulated *in vitro* with C-RBC antigen, when used to treat HeLa cell cultures reduced their incorporation of ^{14}C-labeled amino acids (algal protein hydrolysate, New England Nuclear, Boston, Mass., NEC 233) by 76% in a 20-min pulse compared to control supernatants from unstimulated cultures.

The blast transformation of peripheral lymphocytes in the presence of specific antigen is undoubtedly the result of a combination of interrelated events which may be involved in the synthesis of the variety of macromolecules which have been reported in recent years to play a role as the mediators of delayed hypersensitivity (5). The demonstration of a direct link between the immunologically induced blast cell and any of these factors has been difficult to achieve. It has been equally difficult to demonstrate a direct link between the last transformed lymphocyte and antibody synthesis, and some controversy has arisen through the years as to whether or not potential antibody-forming cells circulate (28). The present data adds further evidence to suggest that the answer to both of these questions is yes. In appropriately hyperimmunized rabbits, it is possible to demonstrate cells in the peripheral circulation capable of responding *in vitro* with the synthesis of specific antibody to C-RBC. In the experiments described these constituted less than 1% of the total blast transformed lymphocytes observed in cells from these animals cultured for 4 days in the presence of antigen. The activation of these cells was observed directly by combined plaque and autoradiographic techniques.

Summary. Cultures of peripheral blood lymphocytes from rabbits hyperimmunized with chicken erythrocytes to favor either antibody formation without delayed hypersensitivity, or the latter immune response without significant antibody formation were examined for activated cells producing antibody. A micromodification of the hemolytic plaque technique was coupled with autoradiographic observations to detect significant numbers of IgG antibody-forming cells in the cultures from antibody-forming animals. These cells were observed in cultures maintained for 4 days either in the presence or absence of the antigen. A similar response could not be detected in cultures from normal rabbits or rabbits showing delayed hypersensitivity but no antibody response to the antigen. It was possible, however, to demonstrate lymphotoxin, skin sensitizing factor, and migration inhibitory factor in culture supernatants from delayed hypersensitive animals. A direct link between a small subpopulation of blast transformed peripheral lymphocytes and antibody-forming cells has thus been demonstrated.

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