ON THE NUMBER AND NATURE OF ANTIGEN-SENSITIVE LYMPHOCYTES IN THE BLOOD OF DELAYED-HYPERSENSITIVE HUMAN DONORS

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Considerable progress in our understanding of the basic mechanisms of delayed-type hypersensitivity has been made in recent years, largely as a consequence of studies in vitro (1, 2). Although most of these studies have approached the problem primarily at the phenomenological level, they have established that the immunological information necessary for the initiation of cell-mediated immune reactions in vitro is possessed by the small lymphocyte which, upon interaction with specific antigens can influence, directly or indirectly, a variety of other cells in its environment.

In order to study the cell-mediated immune response on a quantitative basis, we have recently developed a plaque assay which permits determination of the number of antigen-sensitive cells within a population of lymphoid cells (3). The method was designed to detect intrinsic changes produced in sensitized lymphocytes as a consequence of activation by antigen, rather than to measure products secreted by such cells. The basis for our approach is that unstimulated lymphocytes generally do not support replication of viruses, whereas antigen-activated lymphocytes do so.

The quantitative approach to delayed-hypersensitivity reactions in man is of interest at two levels. Because of their importance in the rejection of allografts and tumors, and in the course of some "autoimmune" diseases, there is an urgent need for quantitative measurements in vitro of cell-mediated immunity at the clinical level. We can hardly imagine a clinically useful analysis of an immune response mediated by antibodies if it could be demonstrated only by means of the wheal-and-flare reaction. Yet skin tests are still the principal measure of delayed hypersensitivity in man. Secondly, a method that permits enumeration of specifically sensitized cells is of obvious value for approaching an understanding of the basic mechanisms underlying the delayed-

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type hypersensitivity response. It is perhaps germane to recall the insights into the mechanism of antibody formation which have been provided by the Jerne plaque assay.

In this report we describe the successful application of the virus plaque technique to the enumeration of antigen-sensitive cells in the peripheral blood of tuberculin-sensitive human donors.

Materials and Methods

Antigens.—All in vitro work was done with excipient-free, purified protein derivative of tuberculin (PPD)[1] generously provided by the Ministry of Food, Agriculture, and Fisheries, Weybridge, Surrey, England. Skin tests were carried out with PPD from Parke, Davis and Co., Detroit, Mich.

Peripheral Blood Lymphocytes.—

Donors: The blood used in these experiments was donated by healthy human subjects. According to their dermal reactivity to tuberculin they were grouped as follows: (*a*) Donors who responded with skin reactions (erythema and induration) greater than 10 mm in diameter to intradermal injections of 0.00002 mg of PPD (first test strength). (*b*) Donors who developed delayed skin reactions of similar size only when tested with 0.0001 mg of PPD (intermediate test strength). (*c*) Donors whose skin reactions to 0.0001 mg of PPD measured less than 6 mm in diameter (tuberculin-negative).

Preparation of peripheral blood lymphocytes: 1 unit of whole blood (470 ml) was drawn by the staff of the New York Blood Center into a heparinized sterile plastic bag (Abbott No. 4686, Abbott Co., Miami, Fla.). The final concentration of heparin was 4.5 units/ml of blood. The bag was immediately attached to a Fenwal Leuko-pak nylon filter (Fenwal No. 4 C 2401, Fenwal Laboratories, Morton Grove,Ill.), and blood was allowed to flow by gravity into another sterile plastic bag (Fenwal Transfer-pak, No. 4 R 2022) at 37°C over a period of 10–15 min. To the filtered blood additional preservative-free heparin (10 units/ml, Organon. West Orange, N.J.) was added and the blood was drawn into 250 ml plastic syringes.

The syringes were inverted and the erythrocytes were allowed to sediment at 37°C until either 35–40% of plasma had separated or 1½ hr had elapsed. The plasma was expelled from the syringe into a 125 ml siliconized centrifuge tube and was centrifuged at 200 *g* for 10 min at 4°C. The plasma was removed and the cells were washed in Eagle's minimal essential medium (MEM) (Earle's base) containing 100 units/ml of penicillin and 100 μg/ml of streptomycin and supplemented with 10% fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N.Y.). The preparations were estimated to contain between 87 and 95% lymphocytes.

Culture of Lymphocytes.—The lymphocyte suspensions were adjusted to a density of 2×10^6 viable cells/ml in the culture medium (MEM containing 10% fetal calf serum) and distributed in 1 ml volumes into 12 × 75 mm polypropylene tubes (No. 2063, Falcon Plastics, Los Angeles, Calif.). Paired cultures were made in the presence or absence of antigen (PPD, 5 μg/ml).

In five experiments, 1 μg/ml of vinblastine (Velban, Eli Lilly and Co., Indianapolis, Ind.) was added to control and PPD-stimulated cultures to inhibit mitosis. In these same experi-

[1] *Abbreviations used in this paper*: B cell, bone marrow-derived cell; FCS, fetal calf serum; MEM, Eagle's minimal essential medium; PFU, plaque-forming units; PPD, purified protein derivative of tuberculin; T cell, thymus-derived or thymus-dependent cell; VSV, vesicular stomatitis virus.

ments, parallel cultures were established under the same conditions for studying mitogenesis by means of incorporation of ^{14}C-thymidine. All cultures were incubated vertically at 37°C in an atmosphere of 10% CO_2 in humidified air.

Virus.—Frozen stock of vesicular stomatitis virus (VSV), Indiana strain, was used. For production of virus in quantity confluent monolayers of chicken embryo fibroblasts in MEM supplemented with 10% FCS were infected with virus at a multiplicity of 1. Culture supernatants were harvested at 24 hr, centrifuged at 900 *g* to remove cell debris, frozen in 10% dimethyl sulfoxide, and stored at −70°C until used. The titer, determined after thawing, was approximately 10^9 plaque-forming units (PFU) per milliliter.

Anti-VSV Antisera.—Hartley guinea pigs (Hazelton Laboratories, Baltimore, Md.) were injected in the footpads and necks with 1 ml containing 10^9 PFU of VSV (grown in L-cells) emulsified with complete Freund adjuvant. At various intervals blood samples were obtained and the sera tested for anti-VSV activity by plaque-neutralization tests. When the antibody titer was found to be sufficiently high (0.01 ml of serum completely neutralizing 5×10^7 PFU), the animals were exsanguinated, the sera were pooled, inactivated at 56°C for 30 min, and stored at −70°C.

Preparation of Virus-Susceptible Target Cells.—L-929 mouse fibroblasts were cultivated continuously in spinner bottles in suspension medium (Joklik-modified MEM, Grand Island Biological Co.) supplemented with 5% FCS. For assay of the virus and infectious centers, monolayers of target cells were prepared by plating 2.0×10^6 cells in 5 ml of MEM (Earle's base) containing 6% FCS in 60 mm plastic Petri dishes (Falcon Plastics No. 3002) 24 hr before use, and by incubating them at 37°C in 10% CO_2 in air. At the time of assay the L-cells had formed confluent cell monolayers.

Assay for Virus-Producing Cells.—After culture for various periods of time with or without PPD, the lymphocytes were washed twice by centrifugation at 250 *g* for 10 min and resuspended in 0.2 ml of culture medium. The cells were infected with VSV at a multiplicity of 50 and incubated at 37°C for 2 hr to permit virus adsorption and penetration.

Excess virus was removed by washing the cells twice, and the remaining virus was neutralized by exposure to 0.01 ml of anti-VSV serum for 1 hr at 4°C. The lymphocytes were then washed three times, resuspended in MEM containing 6% FCS, and counted in a Coulter particle counter (Coulter Electronics, Hialeah, Fla.) with a 100 μ aperture.

The monolayers of L-cells were drained and one of several dilutions of virus-infected lymphocytes was added in 0.2 ml volumes. Next 1 ml of 1% warm agar (Ionagar No. 2, Oxoid, Colab Laboratories, Inc., Chicago Heights, Ill.) in 6% FCS plus MEM, maintained at 45°–47°C, was mixed with the lymphocytes by quickly expelling, withdrawing, and re-expelling the entire volume with a warm 1 ml pipette. When this layer of agar had solidified (approximately 10 min) another layer of 1.5 ml of warm agar was added to provide further nutrients. Each cell sample was plated in three dilutions and each dilution plated in duplicate. For detecting the possible presence of free virus, samples of the supernatant, after removal of the virus-infected lymphocytes by centrifugation, were routinely plated.

The plates were incubated at 37°C in 10% CO_2 in air for 2 days, then vitally stained with 1.5 ml of neutral red (1:10000) in Hanks' solution. After 1–3 hr of incubation, the unstained plaques of lysed target cells were clearly visible against the red background of stained viable cells. The results, after subtracting plaques from supernatants, were recorded as PFU per 10^6 cells plated.

Determination of Thymidine Incorporation.—Cultures to be tested for mitogenesis received 0.1 μCi of 2-^{14}C-thymidine (specific activity 51.8 mCi/mM, Schwarz Bio Research, Inc., Orangeburg, N.Y.) 6 hr before assay. The cells, after chilling in an ice bath, were retained on a 0.22 μ membrane filter (GSWPO 25, Millipore Corp., Bedford, Mass.), washed twice with cold isotonic saline, and the acid-insoluble fraction was then precipitated on the filter with

three 10 ml volumes of 5% trichloracetic acid. The filter containing the precipitate was placed under 10 ml of scintillation fluid (containing a mixture of 32 ml Permafluor [Packard Instrument Company, Downers Grove, Ill.]) in Triton-X 100 and toluene (1:2) and the disintegrations were counted in a Tracerlab scintillation counter (Tracerlab Div., Richmond, Calif.) at an efficiency of 75%.

TABLE I

Virus Plaque Formation by Human Lymphocytes Cultured in Medium with or without Antigen

Results of skin test with PPD	PFU per 10^6 cells		Δ PFU per 10^6 cells	Average Δ PFU per 10^6 cells
	Medium			
	Control	PPD		
				±SE
Negative	480	362	−118	
(intermed. test strength)	3300	2,245	−555	
				−337 ± 218
Positive	750	8,600	7856	
(intermed. test strength)	300	4,890	4590	
	345	2,700	2355	
	90	890	800	
	325	3,300	2975	
	1095	4,285	3190	
				3627 ± 893
Positive	1218	2,767	1549	
(first test strength)	412	2,835	2423	
	3780	12,868	9088	
	2198	6,075	3892	
	3768	6,057	2289	
	2295	4,630	2335	
				3596 ± 1144

RESULTS

Purified peripheral blood lymphocytes from the three groups of donors were cultured in the presence or absence of antigen (PPD) in 14 experiments. After various periods of time the cells were removed, infected with VSV, and plated in the plaque assay to permit enumeration of virus-producing cells. The individual results of both control and PPD-stimulated cultures of 14 donors after 3 days are presented in Table I. While the background of virus-producing cells in control medium varied, there was a significant increase in the number of plaque-forming cells in the PPD-stimulated cultures of lymphocytes obtained from hypersensitive donors in every experiment. The lymphocytes of tuberculin-negative donors in two control experiments failed to produce an increase

in the number of plaques above control levels after cultivation with PPD. The difference between the numbers of PFU in cultures with and without antigen (ΔPFU) is assumed to represent the number of antigen-sensitive cells in the population. Interestingly, lymphocytes obtained from donors with positive skin reactions to intermediate strength PPD (0.0001 mg) or to first test strength (0.00002 mg) gave quite similar values for antigen-sensitive cells in this assay. The mean maximum number of antigen-reactive cells observed on day 3 was $3.6 \pm 1.0/1000$ cells plated (Table I).

In previous studies of the rate of activation of sensitized guinea pig lymph node lymphocytes by PPD it was found that the increase was linear over a 4 day period (3). Inhibition of cell division by vinblastine, colchicine, or thymidine blockade failed to lower significantly the number of plaque-forming cells observed after 2 days of stimulation. Unfortunately, interpretation of the data in these studies obtained after 48 hr of culture was complicated by the low viability in the cell cultures. The better survival of human lymphocytes in culture afforded a superior system for analyzing the kinetics of plaque formation.

In five of these experiments lymphocytes from hypersensitive donors were cultured with PPD in the presence or absence of the mitotic inhibitor vinblastine (Velban) before virus infection and assay. Parallel cultures of the same cells were analyzed on days 2–4 by means of both the virus plaque assay and the determination of ^{14}C-thymidine incorporation into DNA as an index of mitotic activity. The results are shown in Fig. 1. The increase in the number of plaque-forming lymphocytes after antigenic stimulation was approximately linear, as was the case with guinea pig lymph node cells. Further, the presence of the mitotic inhibitor had no effect on the response. (Fig. 1 A).

In contrast, the increase in thymidine incorporation after antigenic stimulation (Fig. 1 B) was nonlinear and possibly exponential. It is clearly evident that vinblastine was effective in totally suppressing antigen-induced mitotic activity under the conditions of the experiment. Indeed, the thymidine incorporation by the antigen-stimulated cultures was less than that of the control cultures, perhaps indicating that many cells in the antigen-stimulated culture entered the mitotic cycle and were then killed by the effects of the mitotic inhibitor.

DISCUSSION

Two types of in vitro systems have been used previously to assay cell-mediated immune responses. The first measures the ability of antigen-activated lymphocytes to exert a direct cytotoxic effect on a variety of target cells (4, 5). The second type depends upon detection of soluble products produced by antigen-stimulated lymphocytes (1). While both these approaches hold promise, they have not yet provided sensitive and reproducible in vitro assays for cell-mediated immunity in man.

The virus plaque assay used here depends upon a change in sensitized lymphocytes, brought about by specific antigens, which enables them to support the replication of RNA viruses. Studies in several laboratories had previously shown that, while normal lymphocytes were essentially unable to replicate viruses, lymphocytes activated by phytohemagglutinin produced substantial

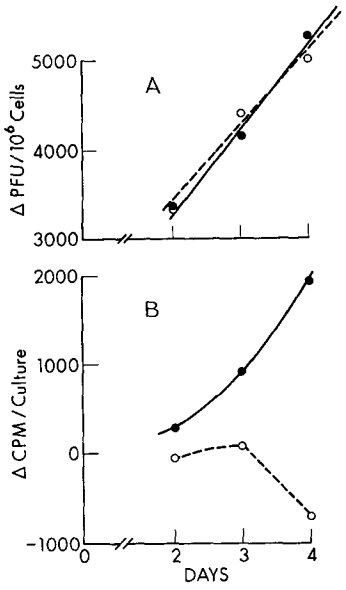

FIG. 1 A. Effect of vinblastine on the increase in antigen-sensitive cells (ΔPFU) detected by the virus plaque assay after PPD stimulation. ●—● indicate the average for five donors cultured in the absence of mitotic inhibitor; ○—○ indicate the results of the same donors' cells cultured in the presence of vinblastine (1 μg/ml).

FIG. 1 B. Effect of vinblastine on the incorporation of ^{14}C-thymidine into DNA following PPD stimulation. ●—● indicate the average increase in incorporation for paired cultures from the same five donors as above in the absence of mitotic inhibitor; ○—○ indicate the average results of the same donors' cells cultured in the presence of vinblastine (1 μg/ml).

yields (6–8). Our own work indicated that this is also the case when sensitized lymphocytes are activated by antigen. Lymphocytes from tuberculin-sensitive guinea pigs stimulated by PPD allowed replication of two different RNA viruses, Newcastle disease virus and vesicular stomatitis virus, to essentially the same degree, indicating that the number of virus plaques observed reflects a property of the activated lymphocytes and not of the virus used (3).

The correlation of the assay with delayed-type hypersensitivity was demonstrated by two types of control experiments. Lymphocytes from normal, nonsensitized animals produced no increase in the number of plaques after culture

with PPD. Furthermore, lymphocytes from guinea pigs that had been immunized with PPD adsorbed onto alumina to produce circulating antibodies and not possessing delayed-type hypersensitivity, did not produce an increased number of viral plaque-forming cells upon culture in vitro with PPD.

As in the Jerne assay for antibody-producing cells, there is a background of virus plaques produced by the cells of nonstimulated control cultures. When this background is subtracted from the number of plaques observed after antigenic stimulation, the increment in plaque-forming cells (ΔPFU) has been taken to represent the number of antigen-sensitive cells in the population.

The present results indicate that the virus plaque assay can be successfully applied to the study in vitro of delayed-type hypersensitivity in man. In the case of most highly skin-reactive indivuduals who were positive to first test strength PPD, we observed a linear increase of virus-producing cells, as in the guinea pig, rising to a maximum of approximately 6/1000 cells on the 4th day of culture with PPD (Fig. 1 A). This figure is somewhat lower than the maximal number of antigen-sensitive cells detected in lymph node cell populations of exquisitely sensitive guinea pigs (approximately 20/1000 cells) but interestingly it is of the same order of magnitude as that detected in mixed cultures of lymphocytes from two normal, unrelated donors are cocultivated (9, 10). That donors with skin reactions to intermediate or to first strength PPD had approximately equal numbers of virus plaque-producing lymphocytes suggests that differences in skin reactivity could result from secondary events at the test site rather than from differences in the numbers of committed lymphocytes.

Perhaps the most surprising aspect of the results is the apparent linearity of the increase in ΔPFU as the cells are cultured with antigen over a 4 day period. From other studies we might have expected an exponential increase. In careful cinemicrographic studies of blast cell transformation of sensitized human peripheral lymphocytes by antigens, Marshall et al. observed an exponential increase in the number of transformed cells after a latent period of about 48 hr (11). Numerous studies of the kinetics of production of antibody plaque-forming cells in the Jerne assay have shown, both in vivo and in vitro, that the production of antibody-forming cells generally follows exponential kinetics (12–14).

There are at least two plausible explanations for our findings. First, it is possible that the plaque-forming cells in this assay are in fact dividing, but because of imperfect culture conditions, as some cells divide others may die; thus, the linear relationship observed would be merely fortuitous. Secondly, we may be detecting a cell which is distinct from the cell measured in blast cell transformation or in antibody formation by virtue of its being nondividing under the conditions of our experiments. We have tested the former possibility by using vinblastine, an irreversible inhibitor of mitosis (15). The results shown

in Figure 1 A clearly indicate a linear increase in the number of viral plaque-forming cells, which is unaffected by the presence of the mitotic inhibitor. In previous work with guinea pigs, thymidine blockade and colchicine were employed in addition to stop mitosis; the results were essentially the same (3).

That vinblastine under the conditions employed was effective in inhibiting mitosis is demonstrated by the results shown in Figure 1 B. Blastogenesis in parallel cultures, as detected by thymidine incorporation, was abrogated when vinblastine was added. Preliminary experiments have indicated that two cells are involved in the activation of lymphocytes by antigen to produce virus, and the slow increase in plaque-forming cells probably reflects the time required for cell interaction.[2] We are thus led to the conclusion that the cell measured by the virus plaque assay is essentially a nondividing cell, at least during the period of the test, and that it is likely to be distinct from the antibody-producing cell or the blast cell. However, it is clear that it must be derived from dividing precursors and it cannot be excluded that, at a later stage or in a different anatomic compartment, it might become again a dividing cell.

It is our view that the antigen-sensitive cell in this system may well be the effector cell which mediates delayed-type hypersensitivity reactions in vivo. There are at least five lines of evidence indicating that this effector cell does not divide: (*a*) There is little or no indication that significant numbers of lymphocytes proliferate at the reaction site (16). (*b*) Many delayed-type reactions in vivo reach their maximum at 24–30 hr, declining after 36–48 hr. This is in contrast to the mitogenic response of human lymphocytes following antigenic stimulation in vitro which does not begin until approximately 48 hr and proceeds to a maximum in 6–7 days, long after in vivo reactions would have disappeared. (*c*) In passive transfer experiments in the guinea pig (17), sensitized lymphocytes treated with low doses of mitomycin C, under conditions where cell division would be blocked, were perfectly capable of effecting transfer of contact and tuberculin hypersensitivity reactions. (*d*) While the induction of cell-mediated immunity to *Listeria monocytogenes* infection in mice requires division, once cellular immunity is acquired it is completely resistant to treatment with vinblastine in vivo (18). (*e*) The delayed-type hypersensitivity response is much less sensitive to radiation than antibody formation (19). It thus appears likely that the effector cell mediates the delayed-hypersensitivity reaction directly without dividing.

On the basis of work done in many laboratories, we may summarize the characteristics of the cell involved in mediating delayed-type hypersensitivity reaction as follows: (*a*) It is a thymus-derived or thymus-dependent (T) cell (20–23). (*b*) With respect to hapten–protein conjugates this cell is believed

[2] Jimenez, L., and B. R. Bloom. Unpublished observation.

to have specificity primarily for "carrier" antigenic determinants in contrast to antibody-forming cells which are primarily hapten-specific (24, 25). (*c*) The cell is not believed to be engaged to any significant degree in secretion of antibody. (*d*) It is a radiation-resistant, nondividing cell (see above).

It seems to us that the cell with these characteristics might have a broader immunological function than involvement solely in the effector process in delayed-type hypersensitivity reactions. Present information on the mechanism of production of antibodies suggests that cell cooperation between two cell types is required in at least some systems. One cell type, a bone marrow-derived or B cell, actually synthesizes and secretes the antibody molecules, which are found primarily to have specificity for haptenic determinants. However, before the B cells can differentiate and produce antibodies, interaction with a thymus-dependent cell (T cell) which is responsible for antigen recognition is required (26, 27). This T cell has been shown to be radiation-resistant and, presumably, nondividing (28). Further, in the secondary antibody response, it primarily has carrier specificity (29–31).

In our view the characteristics of the effector cell in delayed-type hypersensitivity cannot be distinguished from those of the thymus-derived helper cell which carries immunological memory in antibody formation and may be the same cell. Upon being engaged by carrier determinants on antigen molecules, this cell may mediate the delayed-type response directly or by means of the various soluble products which it releases such as migration-inhibitory factor, chemotactic factor, lymphotoxin, blastogenic factor, etc. It is tempting to speculate that one factor elaborated by this cell may activate those bone marrow-derived B cells which have been engaged by haptenic determinants, to proliferate and to differentiate into plasma cells and produce antibodies. An intimate relationship between delayed hypersensitivity and antibody formation has certainly been suggested previously by several authors (32–35). The hypothesis, as presented here, has the virtues of serving to unify the mechanisms of the two immune responses as well as being amenable to direct experimental testing.

SUMMARY

The virus plaque assay has been successfully employed to enumerate antigen-sensitive cells in the peripheral blood lymphocyte populations of tuberculin-hypersensitive human donors. The method is based on the finding that, while resting lymphocytes are unable to produce a variety of viruses upon infection, lymphocytes activated by specific antigens become capable of virus replication. The average number of antigen-sensitive cells detected in cell populations from donors reacting to first test strength or intermediate test strength tuberculin was approximately 3.6/1000 lymphocytes, and the averages for both groups were similar.

Studies on the kinetics of appearance of these virus plaque-forming cells and

on the effects of the mitotic inhibitor, vinblastine, indicate that the activation of these antigen-sensitive cells is a linear process and that the cells must be nondividing cells during this process. These qualities contrast markedly with those described for the mitogenic response and the antibody-producing cells which require cell division and increase exponentially. On the basis of these experiments it is suggested that the antigen-sensitive cell measured in the virus plaque assay is the effector cell in delayed-type hypersensitivity reactions and, in addition, may be one of the cells critically involved in antibody formation.

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