A PLAQUE ASSAY FOR ENUMERATING ANTIGEN-SENSITIVE CELLS IN DELAYED-TYPE HYPERSENSITIVITY*

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In recent years our knowledge of the mechanism of delayed-type hypersensitivity reactions has been greatly enhanced by the study of sensitized cells in vitro (1). From these studies it has been established that it is the small lymphocyte which possesses the immunological information for the reaction and which is capable of influencing a variety of other cells in its environment, either directly (2, 3) or by means of a variety of soluble factors. These include the migration inhibitory factor (MIF)[1] (4, 5), skin reactive factor (6, 7), chemotatic factor (8), lymphotoxin (LT) (9), a blastogenic factor (10–12), interferon (13), and antibodies (14). While it is hoped that some of these molecules, at least, will prove to be mediators of the delayed-type hypersensitivity reaction in vivo, the studies of these factors have not been able to provide any insight into a fundamental problem, namely, the number of specifically sensitized cells involved.

In contrast, one has only to reflect upon the enormous strides made in understanding the mechanisms of antibody formation as a direct consequence of the Jerne plaque technique (15) to recognize the importance of having a method available for enumerating specifically sensitized cells in delayed hypersensitivity. This paper will present a novel method for enumerating specific antigen-sensitive lymphocytes obtained from lymph nodes of guinea pigs sensitized to tuberculin by complete Freund's adjuvant. The approach was designed to detect intrinsic changes in sensitized lymphocytes activated by antigens, rather than to measure products of this interaction which may be present only in minute amounts.

The fundamental property of antigen-sensitive cells that is measured in these experiments is the ability of such cells to permit replication of RNA viruses. It is well known that resting lymphocytes are quite refractory to infection by a

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[1] *Abbreviations used in this paper:* CEF, chicken embryo fibroblasts; MIF, migration inhibitory factor; NDV, Newcastle disease virus; PFU, plaque-forming units; PHA phytohemagglutinin; PPD, purified protein derivative; VSV, vesicular stomatitis virus.

great number of viruses which are capable of replication in most other mammalian cells (16). When such lymphocytes are "activated" by phytohemagglutinin (PHA) it has been observed that they become capable of permitting a variety of viruses to replicate, including measles (17), ECHO virus (18), herpes simplex (19), mumps (20), polio (21), and vesicular stomatitis virus (VSV) (22, 23). When we adapted this type of experiment to tuberculin-sensitive lymphocytes, we found that the same principle applied—namely, that the cells activated by specific antigen become capable of replicating viruses. After various times of antigenic stimulation, virus was adsorbed and, after the free virus was washed and neutralized , the cells were plated in agar over a target cell monolayer highly susceptible to the virus. Wherever an activated lymphocyte yielded virus, a discrete plaque in the monolayer known as an infectious center was seen. The increase in plaques above the background nonactivated control lymphyocytes was taken to represent the number of antigen-sensitive cells in the population.

Materials and Methods

Animals.—The National Institutes of Health guidelines for handling of experimental animals were followed in all experiments. Random-bred Hartley guinea pigs purchased from commercial breeders were used in most experiments. In a few experiments, Rockefeller University guinea pigs, kindly given by Dr. M. W. Chase, were employed.

Sensitization.—Animals were sensitized to tuberculin by the inoculation of complete Freund's adjuvant (3.3 mg/ml $H_{37}Ra$ *Mycobacteria*, Difco Laboratories, Inc., Detroit, Mich.) on one occasion into the four footpads and into the nuchal muscles (4). 3–4 wk later, animals were tested intradermally with 10 μg of tuberculin purified protein derivatives (PPD, Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge, England). Animals showing reactions of 15 mm or greater were used as cell donors. However, testing is not required since, on occasion, untested donors were used with results identical to tested animals.

For immunizing guinea pigs to produce circulating antibodies to PPD in the absence of delayed-type hypersensitivity, the regimen described previously (4) was employed. PPD (1.25 mg) in saline was adsorbed to alumina (approximately 7.5 mg), and after incubation at 37°C for 30 min with occasional stirring, the material was inoculated into all four footpads and the nuchal muscles. The animals were tested as described above.

Collection and Culture of Lymphocytes.—Lymph nodes were obtained from the brachial, axillary, cervical, inguinal, and popliteal regions, trimmed of fat, and the lymphocytes were expressed by gentle teasing with stainless steel rakes as described previously (6). The cell suspension was filtered through 40- and 100-mesh stainless steel screens to remove clumps, and was washed three times in Hank's solution by centrifugation at 250 *g* for 10 min. The cells were resuspended in Eagle's minimal essential medium (MEM) containing 100 units/ml penicillin and 100 μg/ml streptomycin plus 5% normal guinea pig serum. From this cell suspension, 1.5 ml (or 0.5 ml) volumes were cultured in large (or small) Leighton tubes (Bellco Glass Inc., Vineland, N. J.) at a viable-cell density of 15×10^6 cells/ml. In every experiment parallel cultures were set in normal medium and medium containing antigen, PPD (25 μg/ml). The cultures were incubated at 37°C in an atmosphere of 7% CO_2 in humidified air.

Cultivation of Virus-Susceptible Cells.

Primary chicken embryo fibroblasts (CEF): 5-day old chick embryos (Cofal negative) were obtained from Spafas Farms. After 2–3 days incubation, the chick embryos were removed and minced. After trypsinizing in 0.25% trypsin solution and filtering through sterile gauze, the cells were washed, plated in NCI medium containing 6% calf serum (Grand Island Biological Co., Grand Island, N. Y.), 7.5×10^6 cells in 5 ml medium per 60 mm Petri dish (Falcon Plastics, Los Angeles, Calif.) and used 3 days later.

L cells: L-929 cells were cultivated continuously in spinner bottles in minimal essential medium (MEM) suspension medium (Joklik-modified, Grand Island Biological Co.) containing 10% fetal calf serum. For the virus assay 2.5×10^6 cells in 5 ml of MEM (Earle's) containing 6% calf serum were plated in 60 mm plastic Petri dishes 24 hr prior to use, and incubated at 37°C in 7% CO_2. In all cases, at the time of assay, the plates used had a confluent monolayer.

Viruses.

Newcastle disease virus (NDV): The preparation of Newcastle disease virus on the chick chorioallantois has been described elsewhere (26). It was stored at −70°C.

Vesicular stomatitis virus (VSV): Frozen stock of the Indiana strain, generously supplied by J. Mudd, was passaged five times on L cell monolayers, the multiplicity being selected each time for maximum cytopathic effects. The most effective dilution in the last passage was used to produce virus in quantity. After infecting the monolayers, culture supernatants were harvested at 24 hr, centrifuged at 1500 rpm for 15 min to remove debris, and frozen at −70°C, either directly or after the addition of 10% dimethyl sulfoxide (DMSO). All stock virus was titrated after thawing to determine number of plaque-forming units (PFU), and tubes not containing DMSO were thawed only once.

Infection of lymphocytes with virus: After the lymphocytes had been cultured in the presence or absence of PPD for various periods of time, they were washed three times, resuspended in 0.2 ml of MEM containing 6% calf serum, infected with virus at a multiplicity of 3 for VSV and 50 for NDV, and incubated at 37°C in 7% CO_2 for 2 hr. The excess virus was then removed by three further washings and free virus remaining was neutralized with 0.01 ml of the corresponding inactivated, guinea pig anti-viral antiserum by incubation for 1 hr at 4°C. The cells were washed three times more and resuspended in MEM containing 6% calf serum. Cell counts were made in a Coulter particle counter with a 100 μ aperture. Neutralization of free virus by antiserum did not cause spurious lymphocyte activation and could be omitted if larger wash volumes and more extensive washings were performed.

Plaque assay for virus-producing cells: The monolayer of virus-susceptible cells was drained of medium, and dilutions of virus-infected lymphocyte suspensions in 0.3 ml were added. Next, 1 ml of 1% agar (Oxoid, Ionagar No. 2) in MEM containing 6% calf serum, rigorously maintained at 47–49°C, was added and mixed with the lymphocytes by quickly withdrawing and expelling the entire volume with the warm 1 ml pipette. When this had solidified, a second 1.5 ml agar layer was poured over the first as a nutrient layer. The plates were incubated at 37°C in 7% CO_2 for as long as required for plaques to form, i.e. 2 days for VSV in L cells; 3 days for NDV in CEF. Each sample was plated in three dilutions with each dilution made in duplicate or triplicate.

At the end of the incubation period, the plates were vitally stained with 1.5 ml of 1:10,000 neutral red in Hank's solution. After 1–3 hr of incubation, the clear plaques of lysis were seen against the red background of healthy cells which had taken up the neutral red.

In all cases, the results were expressed as PFU/10^6 cells actually plated.

RESULTS

Initial Experiments: Measuring Plaques Produced by Lymphocytes Infected with Newcastle Disease Virus.—Lymph node lymphocytes obtained from tuberculin

hypersensitive guinea pigs were cultured in Leighton tubes in the presence and absence of PPD under conditions which had previously been shown to initiate the production of the migration inhibitory factor (MIF) (4, 6). To avoid complications due to mixed lymphocyte reactions, cells from individual donors only were examined, never pools. In the first series of experiments the cells were infected with Newcastle disease virus (NDV) 24 and 96 hr after stimulation by antigen, and plated on chick embryo fibroblasts. The multiplicity of infection was 50–100 and the number of cells plated varied from 6×10^4 to 2.5×10^6. All assays were performed in duplicate or triplicate. In each experiment controls included: (a) frozen and thawed lymphocytes which, because of the long eclipse period of the virus in lymphocytes, represented a good approximation of infectious centers derived from non-eclipsed virus, that is, the number of cells which have taken up but not uncoated the virus; and (*b*) the supernatant of the final cell suspension, which indicates the number of plaques due to free viruses not completely removed by washing nor neutralized by antiserum. The results of eight experiments are shown in Table I. It is clear that the number of plaque-forming cells varies from animal to animal but there is a discernible increase in antigen stimulated cells to become infectious centers and produce plaques even at 24 hr. By 96 hr of culture with antigen (PPD) there was a very large increase of antigen-activated lymphocytes producing plaques relative to controls. At 24 hr the average increase in infectious centers of antigen-stimulated lymphocytes relative to control lymphocytes was $331/10^6$ cells; by 96 hr the increase was $9852/10^6$ cells. In one experiment cells from a normal, nonhypersensitive animal were cultured with and without antigen and plated at 96 hr; there was no spurious nonspecific stimulation to produce plaques.

Kinetics of Plaque Formation: Antigen-activated Lymphocytes Infected with Vesicular Stomatitis Virus and Plated on L-Cells.—If the increase in plaques represents a change in a fundamental property intrinsic to the lymphocytes, then the relative increase in plaque formation should be manifested by infection of lymphocytes by a variety of viruses. Because of the inconvenience and expense of preparing monolayers of primary chick embryo cells and their inevitable lack of uniformity from experiment to experiment, we chose to employ mouse L cells as the target cells and used VSV as our indicator virus. The L cells used for these experiments have the added convenience of growing routinely in spinner culture, but readily forming monolayers when plated in appropriate medium. Sensitized lymphocytes were cultured and stimulated with PPD as before. At various times after antigen stimulation, they were infected with VSV at a multiplicity of 3–10, as were nonstimulated control lymphocytes. Assays were performed in triplicate. The number of cells plated varied from 3×10^5 to 3×10^3. Plaques were easily distinguished 36–40 hr after plating. The results of 15 experiments are illustrated graphically in Fig. 1. It is readily apparent that the number of lymphocytes capable of replicating VSV increases after antigen stimulation, while the

ability of aliquots of sensitized lymphocytes not stimulated in vitro with antigen remains constant. It is not surprising that there is a background of cells which replicate viruses in these lymph node populations. Nevertheless, as in the Jerne plaque assay, it would be expected that the number of these background cells would be the same in antigen-stimulated and in control, non-stimulated samples. The number of antigen-sensitive cells (or their progeny),

TABLE I

Plaques Produced by Lymphocytes Cultured with and without PPD and Infected with NDV

Time of infection	PFU/10^6 cells		Δ PFU/10^6
	Control	Antigen-stimulated	
24 hr			
Sensitized lymphocytes	1,296	1,920	634
	2,000	3,360	1,360
	720	960	240
	220	1,200	980
	360	1,040	680
	69	271	202
	296	405	209
	642	1,084	342
Average			331
96 hr.			
	457	1,266	809
	740	2,510	1,770
	380	3,300	2,920
	154	19,200	11,146
	907	21,250	20,343
	625	22,807	22,184
Average			9,852
96 hr.			
Normal lymphocytes	1,000	820	−180

then, is simply the increment in the number of plaques obtained in the antigen-stimulated cultures above those in the control cultures, and this is plotted in Fig. 2 both in logarithmic and arithmetic scales. It is interesting that the data are most consistent with a linear increase. The average number of antigen-sensitive cells found in the stimulated cultures varied from 1,029/10^6 at 24 hr to 16,689/10^6 at 96 hr. In two experiments no stimulation was seen at 24 hr probably due to technical failure in the experiment; nevertheless, the data are included for the sake of completeness.

Plaque Formation by Lymphocytes from Normal Animals or Animals Immunized to Produce Circulating Antibodies to PPD.—It remained possible for the increase in plaque formation due to antigenic stimulation to represent either

a nonspecific reactivity induced in lymphocytes by PPD or simply the detection of antibody-producing cells, rather than measuring the number of antigen-reactive sensitized cells. To exclude these possibilities, experiments were performed on cells from four normal animals and four animals which were immunized with PPD adsorbed to alumina which had previously (4) been

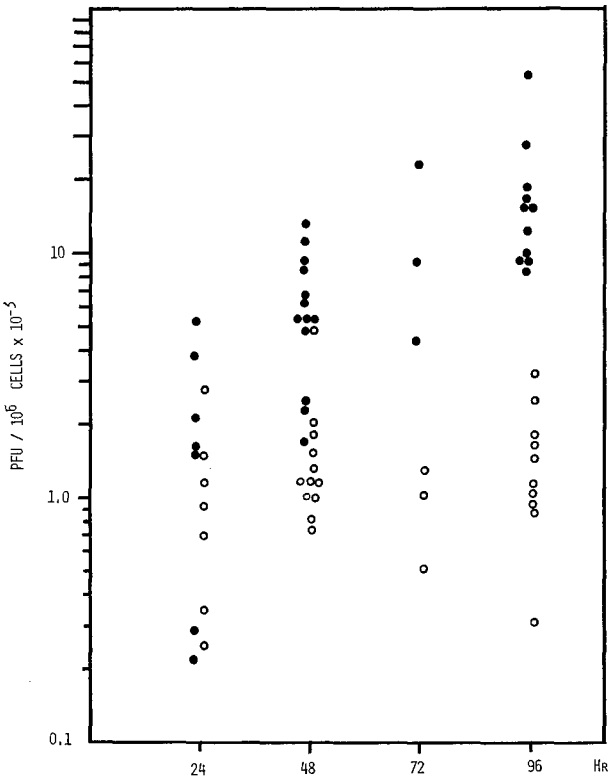

FIG. 1. Vesicular stomatitis virus formation by tuberculin-sensitive lymphocytes cultured in control medium (open circles) or in medium containing PPD (closed circles).

shown to cause production of high titers of hemagglutinating antibodies to PPD. The results of these experiments are shown in Fig. 3. PPD did not cause any significant increase in plaque-forming ability of normal lymphocytes. Nor were lymphocytes from animals that produced antibodies to PPD stimulated in vitro by PPD to replicate VSV.

Inhibition of Cell Division and Determination of Initial Antigen Reactive Cells.—Blast cell transformation of sensitized lymphocytes and antigen-induced stimulation of thymidine incorporation and proliferation have con-

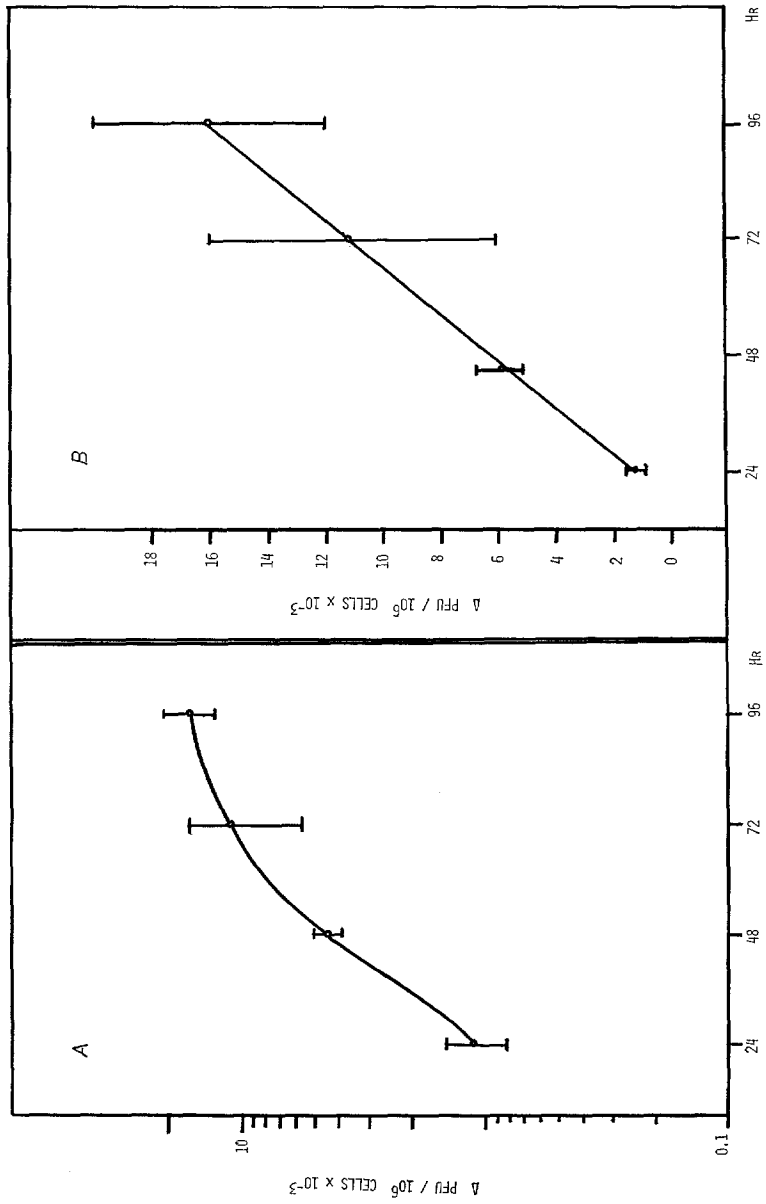

FIG. 2. Kinetics of the increase above background in VSV plaque-forming cells of PPD-stimulated lymphocytes (Δ PFU). The average values from 15 experiments are plotted in *A* on a logarithmic scale and in *B* on an arithmetic scale.

sistently been observed in our cultures (24). While it is not known precisely which cell it is that is capable of replicating virus in these cultures, it is obvious that the increase of plaques could be due either to initial antigen-sensitive cells or to progeny of these cells after division. Since it is known that antigen activation and stimulation of lymphocytes by PHA do not induce synchronous division, we studied the effect of mitotic inhibitors on plaque formation in antigen stimulated and control cultures. The inhibitors chosen for these experiments were colchicine and vinblastine which immobilize the mitotic spindles

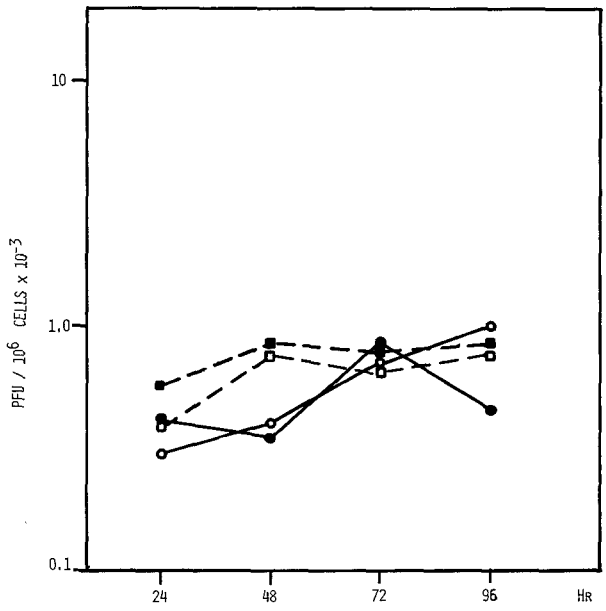

FIG. 3. Vesicular stomatitis virus plaque formation by normal lymphocytes (solid lines) and by lymphocytes from antibody-producing animals (broken lines). The cells were cultured either in control medium (open symbols) or in medium containing PPD (closed symbols).

in metaphase, or thymidine used in sufficient concentration to block conversion of cytidine to deoxycytidine and, thus, DNA synthesis. To assure ourselves that these agents were effective, their ability to block division was tested on L cells. The results of experiments on lymphocyte cells stimulated for 48 hr are shown in Table II. For these experiments the mitotic inhibitor was added with or without antigen at 0 time, and the ability of the cells to produce plaques was tested at 48 hr. There was only very slight suppression of plaque formation at 48 hr with either colchicine, vinblastine, or thymidine. In the concentrations used, and in fact, in greater dilutions, these agents effectively blocked cell division of L cells. While further studies on the kinetics of increase in plaque-

forming cells and the effect of mitotic inhibitors are required, present results suggest that in the first 48 hr the increases in antigen-induced plaques in Fig. 2 represent essentially initial antigen-reactive cells. Preliminary data indicate that those agents are without marked effect even at 96 hr. Thus, it becomes feasible to determine the number of initial antigen-sensitive cells present in a population by means of this technique.

TABLE II

Effect of Mitotic Inhibitors on VSV Plaque Formation of Lymphocytes Cultured with Antigen for 48 Hr

Lymphocytes cultured with	Average PFU/10^6	(No. of experiments)	L cells cultured with	Cell No.* (24 hr)
PPD	5084	7	MEM	3.0×10^5
PPD + colchicine (1–2 μg/ml)	4833	7	Colchicine (1 μg/ml)	0.9×10^5
PPD	7177	3		
PPD + thymidine (5×10^{-3} M)	4830	3	Thymidine (5×10^{-3} M)	1.8×10^5
PPD	3389	3		
PPD + vinblastine (0.5–1 μg/ml)	3414	3	Vinblastine (1 μg/ml)	1.1×10^5

* Tubes were seeded with 1.4×10^5 cells.

DISCUSSION

There is a paucity of information available on the number of specifically sensitized cells in a leukocyte population from individuals with delayed hypersensitivity. While a few attempts have been made to study the binding of labeled antigen to leukocytes obtained from sensitized and normal animals, the results of these experiments have been essentially inconclusive because of the generally high background of nonspecific adsorption of the labeled antigens to the lymphocytes, and differences of binding between normal and sensitized lymphocytes have not always been statistically significant.

The present method is based on three assumptions: (*a*) resting lymphocytes do not replicate certain RNA viruses; (*b*) antigen-activated small lymphocytes are capable of virus replication; (*c*) small lymphocytes activated by antigen to permit viral replication represent antigen-sensitive cells or specifically sensitized cells in delayed-type hypersensitivity. While no study completely rules out the possibility that small lymphocytes may replicate viruses at a very low level, there are a considerable number of experiments which indicate that the yield of infectious virus from fresh nonstimulated lymphocytes infected

with measles virus (17), ECHO virus 9 (18), mumps (20), vesicular stomatitis virus (22), poliovirus (21), herpes simplex (19), and Newcastle disease virus (25) is very low or nil. Using these same viruses in cultures of human leukocytes or lymphocytes stimulated with phytohemagglutinin (PHA), these same workers have found a marked increase in the ability of the lymphocytes to yield viruses. For example, the replication of vesicular stomatitis virus in human leukocyte cultures activated by PHA was enhanced 6–180-fold (22). It is significant to point out that the time of maximum virus yield in these experiments was commonly 24–48 hr after simulation, a time before any detectable cellular division or thymidine incorporation has taken place. Thus, one of the earliest changes occurring after stimulation of lymphocytes must be acquisition of the ability to permit RNA viruses to replicate. However, the studies on virus yield have not themselves shed light on the question of the number of cells so activated.

The results presented here indicate that lymphocytes from tuberculin-sensitive guinea pigs, when stimulated by specific antigen, become capable of replicating two different RNA viruses, Newcastle disease virus and vesicular stomatitis virus. When such antigen-stimulated lymphocytes were infected and plated in agar above a susceptible monolayer of L cells, it was possible to enumerate those cells capable of producing virus. In these studies, this number increased over a 4 day period from approximately 1/1000 to 16/1000. This increase is independent of the particular virus used and presumably reflects a basic cellular change induced in the lymphocyte by contact with antigen. When similar experiments were performed using lymphocytes from normal, unsensitized animals cultured in the presence and absence of PPD, or using lymphocytes from guinea pigs immunized with PPD on alumina which had circulating antibody but not delayed hypersensitivity, there was no increase in plaques caused by the interaction with antigen.

An alternative explanation for our results would hold that there is a membrane change in antigen-stimulated cells resulting in an increased binding of input virus to cell receptors, rather than an actual increase in viral replication. This possibility cannot be excluded, although the addition of antiserum to neutralize adsorbed virus, the subtraction of plaques from frozen and thawed cells from experimental values, and the clear increases in yield in the experiments on related systems mentioned above (17–23), tend to militate against this interpretation.

The dynamics of cell cultures used for these experiments are clearly quite complex. Putative mediators of cellular immunity, migration inhibitory factor and lymphotoxin, are liberated as early as 6 hr after the sensitized lymphocytes come in contact with the antigens. During the entire period of culture, significant numbers of cells are dying, perhaps amounting to 50–70% of the number of cells cultured initially. Concomitantly, we have observed an antigen-induced

stimulation of thymidine incorporation at 96 hr which reached values of 100–200 times that of controls. The morphology of the antigen-stimulated cells at 96 hr revealed a very large proportion of blast cells. By analogy with the increase in plaque-forming cells in the Jerne assay, it was our expectation that the increase of plaque-forming cells in this assay would be exponential (Fig. 2 A). However, when the data were replotted on a linear scale, a straight line was obtained (Fig. 2 B). Extrapolation of this line would indicate the earliest time that the cells become infectable would be approximately 18 hr. Because of the complexity of the events occurring in the culture, the kinetic data presently available do not permit firm conclusions concerning the true nature and significance of the kinetics at this time. For example, since the variability increased at longer culture times, it is possible that the development of plaque-forming cells is indeed exponential, but that the culture conditions become limiting after 48 hr. Alternatively, the linear relationship might be real, indicating a constant rate of recruitment of potential antigen-sensitive cells which are not destined to undergo division. The possible intervention of a recruiting or blastogenic factor (10, 12) or interferon (13) also warrants examination. Nevertheless, it is significant that when sensitized lymphocytes were stimulated in the presence of a variety of mitotic inhibitors, the number of plaques observed at 48 hr differed not greatly from the untreated antigen-stimulated cultures. This result indicates that at least until 48 hr, the number of plaque-forming cells measured must approximate the number of initial antigen-reactive cells in the population. The kinetic relationships after 48 hr suggest that this may hold even to 96 hr.

As in the Jerne plaque assay for antibody-forming cells, there is in these experiments a background of cells which are capable of replicating viruses. The identity of these cells is presently unknown. It is conceivable that our first assumption is not correct, i.e. that some resting lymphocytes are capable of replicating viruses. A second possibility is that some of the background cells may, in fact, not be lymphocytes, but other cell types. Pertinent, for example, are studies on purified cell types from human peripheral blood by Edelman and Wheelock (23) who found that over 90% of the virus which was produced in unstimulated leukocyte cultures was produced by blood monocytes, or cells which could be removed by passage through a glass bead column. A third contributor to background plaques might be expected to be antibody-producing cells, but experiments employing cells from animals immunized to produce only circulating antibodies have indicated no increase in plaque-forming cells over background. Lastly, one must consider that cells stimulated by antigen in vivo shortly before removal of the lymph nodes could contribute to the background. In any of the above mentioned cases, the number of cells producing virus would be expected to be the same in antigen-stimulated and control cultures. Therefore, it is justifiable to substract the plaques produced by non-

stimulated cells from the total produced by the samples cultured with specific antigen to obtain the antigen induced increment, or Δ PFU, which is the number of antigen-sensitive cells.

As the work presented here was under way, there appeared a report by Willems et al. (21) in which the number of cells responding to phytohemagglutinin was studied by infecting the cells with poliovirus at various times after PHA stimulation and plating in agar over susceptible human fibroblasts. The method used was similar to the method employed here. One difficulty with this type of experiment in which PHA is used is, of course, that of agglutination of the cells, which renders the values for cell numbers plated somewhat ambiguous. Nevertheless, it was clearly observed that on the day of maximal blast cell transformation of human leukocytes as many as 10–20% of the total cells plated produced poliovirus plaques and that each infected cell produced on the average 10 infectious particles per day. At earlier times, for example 24 hr, the number of cells which were able to replicate poliovirus approximated 1/100 to 1/1000. Although our values are lower by approximately an order of magnitude, the results of the present experiments and those of Willems et al. using PHA are entirely consistent.

A number of studies have sought to determine the cellular events which occur after stimulation of lymphocytes with phytohemagglutinin. Newsome (27) approached the problem by studying the labeling in human lymphocytes stimulated by PHA after short pulses of tritiated uridine. Cultures were treated with labeled uridine for 1 hr before collection at 3, 24, 52, 73, and 96 hr after exposure to PHA. The results were evaluated by radioautography. At 3 hr, labeling was present in 2% of the lymphocytes. At 24 hr it was present to some degree in 20% of the lymphocytes, but in 2% the labeling was quite heavy. On this basis Newsome suggested that the maximum number of cells initially responding to PHA was about 2%, although he acknowledged difficulty in using this technique when all cells are incorporating uridine. One has, therefore, to select between more and less heavily labeled cells.

In studies on the kinetics of mixed lymphocyte interaction of nonimmunized rat lymphocytes Wilson et al. (28) found that after a latent period of about 40 hr there was a period of cellular proliferation in which, for many hours, the dividing population doubled in every 9–10 hr. In estimating the number of these cells by use of labeled thymidine and radioautography Wilson found that the cumulative number of newly responding cells in the parent (P) vs. F_1 system amounted to 1–3% of the original parental lymphocyte inoculum. In this system then Wilson suggests that the number of antigen-reacting cells would be approximately 1–5%. Similarly, Bach et al. (29), on the basis of limiting dilution studies on responding cells in one-way mixed leukocyte interaction, estimated the frequency of responding cells to be 0.1–1.0%. These are very difficult figures to reconcile with the prediction of the clonal selection theory

(30). For example, in antibody production measured by the Jerne plaque technique, the number of antigen recognition cells is about 10^{-6} to 10^{-4}. In the case of the mixed lymphocyte interaction or chorioallantoic graft vs. host reactions (31), 1 cell in 20 would appear to be competent against any antigen. The dilemma posed is that, after testing any 50–100 antigens, one would theoretically expect all antigen-reactive cells to be exhausted.

The number of reactive cells detected by the present method is an order of magnitude less than some of these indirect estimates for PHA stimulation and mixed lymphocyte interaction. At 48 hr, between 10^{-3} and 10^{-2} antigen-sensitive cells are detected by this method. We may ask whether every antigen-stimulated cell is detected by this assay. The multiplicity of infection has varied from 3 to 100, so that between 95 and 99.99% of the cells in these experiments would be expected to be infected. However, whether or not these cells are capable of producing infectious centers will depend upon the yield of viruses per cell and upon the efficiency of the method. We have tested the latter point by plating infected L cells as infectious centers over a monolayer of L cells, and found an efficiency of between 50 and 60%; that is, under conditions where 95% of the plated L cells were infected, we obtained plaques from 50 to 60% of them. However, it is likely that the yield of viruses from lymphocytes would be lower than that of L cells, and therefore, the efficiency for lymphocytes would be somewhat lower. At any rate, for a given virus and lymphoid source, the efficiency should be constant throughout the experiments.

Although the assay described is rather time-consuming and requires some technical skill, the method is a general one which should be applicable to lymphocytes obtained from a variety of sources and species. While the results correlate with our third assumption, it remains to be rigorously demonstrated that this assay detects the specifically sensitized lymphocyte in the delayed-type hypersensitivity response. The present results amplify the principle that activated lymphocytes are capable of replicating RNA viruses and suggest that more sophisticated virological techniques may be useful in studying the biology of the lymphocyte.

SUMMARY

A general method is described for enumerating antigen-sensitive lymphocytes obtained from individuals having delayed hypersensitivity, in this case from highly tuberculin-sensitive guinea pigs. The method is based on the observation that resting lymphocytes are generally unable to support replication of RNA viruses, whereas antigen-"activated" lymphocytes can.

Lymph node lymphocytes from individual animals were cultured in the presence or absence of tuberculin purified protein derivatives (PPD). After various periods of time, the cells were infected either with Newcastle disease virus or vesicular stomatitis virus, and plated in agar over a monolayer of cells susceptible to the virus. Wherever a lymphocyte yielded infectious virus,

a discrete plaque in the monolayer could be seen. The increase in plaques of the antigen-stimulated cells over the background of the control sample was taken as the number of antigen-sensitive cells in the population. When lymphocytes from normal guinea pigs or from guinea pigs immunized to produce only circulating antibody to PPD were similarly tested, no increase in plaque-forming units (PFU) was observed.

The average increase in PFU due to antigenic stimulation varied from 1 per 1000 lymphocytes at 24 hr to 16 per 1000 lymphocytes at 96 hr. By employing inhibitors of mitosis (colchicine, vinblastine, and thymidine) it was evident that the increase in PFU at least up to 48 hr was primarily due to initial antigen-reactive cells and not their progeny.

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