MACROPHAGE-LYMPHOCYTE INTERACTION

II. Antigen-Mediated Physical Interactions Between Immune Guinea Pig Lymph Node Lymphocytes and Syngeneic Macrophages

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Close physical association between macrophages and lymphocytes is required for the expression of a variety of the physiological processes of lymphoid cells. Thus, the promotion of lymphocyte viability in vitro (1), the functional maturation and differentiation of immature thymocytes in vitro (2), and the enhancement of the bactericidal capacity of macrophages by antigenically stimulated lymphocytes (3) are all accomplished most efficiently when actual physical approximation develops between lymphocytes and macrophages. In addition, macrophage-lymphocyte cooperation is integrally involved in the development of specific immune phenomena, such as the in vitro induction of primary (4, 5) and secondary (6) antibody responses, as well as the antigen-mediated in vitro initiation of immune lymphocyte proliferation (7–10). In these systems, macrophages are thought to be the cells which initially must interact with antigen for effective lymphocyte triggering (10–12). In view of the inhibition of in vitro immune responses noted when lymphocytes and macrophages are separated mechanically, and the demonstration of clustering of proliferating and/or antibody-producing lymphocytes about antigen-bearing macrophages (4, 5, 7, 8, 13), it is likely that the way macrophages present antigen to lymphocytes involves direct physical contact between these two cell types.

We have previously described an in vitro method to assess the physical interaction between lymphocytes and macrophages (14–16). Using that technique we could demonstrate that macrophages have the unique ability to recognize and reversibly bind thymocytes and lymphocytes in the absence of antigen by means of a trypsin digestible, temperature-sensitive, divalent cation-dependent receptor mechanism.

The studies reported here utilize the same in vitro assay system to characterize the effect of antigen on the physical interactions between lymph node lymphocytes obtained from immune guinea pigs and macrophages. We will demonstrate that the presence of antigen in the cultures markedly enhances the degree of observed macrophage-lymphocyte interaction. These antigen-mediated interactions are dependent on the coincidence in the cultures of immune lymphocytes

and syngeneic, but not allogeneic, antigen-bearing macrophages. The overall functional significance of these events is indicated by the induction of DNA synthesis in those lymphocytes bound to the macrophages. Finally, data are presented which suggest that antigen-stabilized macrophage-lymphocyte interaction is not a consequence of lymphocyte activation, but rather precedes it, and thus, is likely to represent an early step in the sequence of events leading to antigen-specific immune lymphocyte proliferation.

Materials and Methods

Animals. Inbred strain 2 or 13 guinea pigs, weighing 300–500 g (Division of Research Services, NIH), were used as sources of all cell preparations.

Media. All cultures were performed in medium RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with fresh L-glutamine (0.3 mg/ml), gentamicin (10 μg/ml), penicillin (200 U/ml), and 10% fetal bovine serum (FBS[1]; Industrial Biological Laboratories, Rockville, Md.). All washing procedures, except where indicated, were performed in Hank's balanced salt solution (HBSS, Tissue Culture Section, NIH) supplemented with 1% FBS.

Antigens. The following antigens were used: tuberculin-purified protein derivative (PPD, Connaught Medical Research Laboratories, Willowdale, Ontario, Canada); dinitrophenyl guinea pig albumin (DNP-GPA) prepared from 1-fluoro-2,4 dinitrobenzene (Eastman Organic Chemicals, Rochester, N. Y.) and guinea pig albumin (Pentex, Inc., Kankakee, Ill.) by a previously described method (17), containing 22.5 DNP groups per molecule of GPA; dinitrophenyl ovalbumin (DNP-OVA) similarly prepared from five times crystallized egg albumin (General Biochemicals Div., Mogul Corp., Chagrin Fall, Ohio) containing 8.0 DNP groups per molecule of OVA; dinitrophenyl GL (DNP-GL) prepared as described (18) by the reaction of 2,4 dinitrofluorobenzene with a copolymer of L-glutamic acid (60%) and L-lysine (40%) with an average mol wt of 115,000 (Pilot Chemical Division of New England Nuclear, Boston, Mass.) containing 5.5 DNP groups per molecule of GL; keyhole limpet hemocyanin (KLH), prepared as previously described (19).

Immunization. Guinea pigs were immunized with solutions of antigen in PBS or PBS alone emulsified with equal volumes of complete Freund's adjuvant (CFA), containing 0.5 mg/ml of killed *Mycobacterium tuberculosis*, H 37 Ra (Difco Laboratories, Inc., Detroit, Mich.). Each animal received 0.1 ml emulsion in each foot pad for a total immunizing dose of 100 μg of antigen per guinea pig.

Cell Collection and Preparation. Peritoneal exudate cells (PEC) were obtained by injecting guinea pigs intraperitoneally with 25 ml of sterile Marcol 52 (Humble Oil and Refining Co., Houston, Tex.) and harvesting the PEC by lavage 4 days later. The cells were washed with centrifugation three times and suspended in RPMI-1640 with 10% FBS for culture.

Lymph node lymphocytes (LNL) were prepared from animals immunized 2–4 wk previously. The draining lymph nodes were removed, teased apart, and pressed through no. 60 mesh wire screens (W. S. Tyler Co., Mentor, Ohio). The resultant cells were then passed over nylon wool/glass bead adherence columns as previously described (20), washed, and suspended in RPMI-1640 with 10% FBS for culture. LNL prepared in this fashion had a viability of >95% by trypan blue exclusion and contained less than one Mφ per 100 lymphocytes.

Culture Technique. Macrophage (Mφ) monolayers were established in microscope slide/tissue culture chambers (two chambers/slide, Lab-Tek Products, Division of Miles Laboratories, Inc., Naperville, Ill.), as previously described (14). In brief, 1×10^6 PEC obtained from unimmunized guinea pigs were cultured in each chamber in 1 ml RPMI-1640 with 10% FBS. After overnight incuba-

[1] *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; DNP-GL, a dinitrophenyl conjugate of a copolymer of L-glutamic acid and L-lysine; DNP-GPA, a dinitrophenyl conjugate of guinea pig albumin; DNP-OVA, a dinitrophenyl conjugate of egg albumin; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; KLH, keyhole limpet hemocyanin; LNL, lymph node lymphocyte; Mφ, macrophage; PBS, phosphate-buffered saline, pH 7.4; PEC, peritoneal exudate cells; PPD, tuberculin-purified protein derivative.

tion at 37°C in an atmosphere of 5% CO_2 and 95% air, nonadherent cells were washed away and the resultant Mφ monolayer immediately used for experiments. This procedure yielded monolayers composed of 1×10^5 (range: 0.7–1.3 $\times$ 10^5) uniformly viable (>95% by trypan blue exclusion) glass-adherent, morphologically typical macrophages for study. These monolayers contained less than one contaminating lymphocyte per 100 Mφ.

Technique of Brief Macrophage Exposure to Antigen. Mφ monolayers were washed and re-equilibrated at 37°C in 0.9 ml of RPMI-1640. One of the above noted antigens in 0.1 ml PBS was added to the Mφ monolayers to yield a final concentration as indicated in the protocol. After a 60-min incubation at 37°C, the monolayers were washed four times to remove antigen not associated with Mφ and immediately used for binding experiments. Control Mφ monolayers were similarly treated except the added PBS contained no antigen. In some experiments, antigen and LNL were added to Mφ monolayers simultaneously and thus, antigen was present continuously during the period of incubation.

Macrophage-Lymphocyte Interaction. Single cell suspensions of LNL were added to the Mφ monolayers in 1 ml RPMI-1640 with 10% FBS. Incubation at 37°C was carried out on a slowly rocking platform (Bellco Glass, Inc., Vineland, N. J.) to insure uniform distribution of the LNL throughout the chamber. At the conclusion of an experiment, unbound cells were washed away by dipping and swirling the slides in three changes of warm HBSS with 1% FBS. The slides were then fixed with 1% glutaraldehyde in modified Tyrodes buffer and stained with buffered Giemsa in acetone for microscopic observation and quantitation.

Autoradiography. The number of LNL incorporating thymidine in the more prolonged Mφ-LNL interaction assays was assessed by adding 2 μCi of tritiated thymidine (6.7 Ci/mM [^{3}H]TdR, New England Nuclear, Boston, Mass.) to each culture chamber for the last 18 h of incubation. After incubation, LNL which were not bound by Mφ were washed away and the slides fixed with 1% glutaraldehyde. Slides were then dipped in the same batch of Kodak NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, N. Y.), dried, and exposed in light-tight boxes at 4°C for 4 days. The slides were then developed in a 1:3 dilution of Kodak D19 developer at 20°C and stained with methyl green pyronine. The percent of LNL bound by Mφ which were incorporating [^{3}H]TdR was determined by counting 500 randomly chosen LNL and scoring them for the presence or absence of associated silver grains. An LNL was considered positive if it contained four or more silver grains.

Results

Effect of Specific Antigen on the Degree of Macrophage-Lymphocyte Interaction Observed After a 1-H Incubation. When LNL were incubated for 60 min at 37°C with Mφ monolayers which had had no antecedent antigen exposure, clustering of LNL about individual glass-adherent Mφ developed as previously described (14, 15). After unbound LNL were washed away and slides prepared, this physical interaction was quantitated by counting the number of LNL in physical contiguity with each of 200 randomly chosen Mφ and expressed as the total number of LNL bound per 100 Mφ. The vast majority of Mφ associated LNL were found adherent to the surface of the Mφ with less than 5% of the bound LNL actually endocytosed. There was no obvious relationship between the morphological characteristics of a Mφ and its likelihood of binding one or more LNL. There was, however, a direct relationship between the number of added LNL and the number bound. All subsequent experiments were performed by incubating 5×10^6 LNL with Mφ monolayers, which resulted in a degree of interaction in the most linear portion of the binding curve. Under these conditions, 72.3 ± 5.5 LNL were bound per 100 Mφ (Table I), with 48.7 ± 1.3% of Mφ binding one or more LNL. The immune status of the animals from which either the LNL or Mφ were obtained was not found to affect the degree of antigen-independent binding observed after a 60-min incubation.

TABLE I

The Effect of Specific Antigen on the Degree of Macrophage-Lymphocyte Interaction Observed after a 1-H Incubation

Macrophage exposure*	LNL bound per 100 macrophages‡
0	72.3 ± 5.5
PPD	75.1 ± 5.6

* Established Mϕ monolayers were exposed to either PPD (100 μg/ml) in PBS or PBS alone for 60 min at 37°C and washed four times before the addition of 5×10^6 LNL obtained from PPD immune guinea pigs.

‡ Data express LNL bound per 100 Mϕ after a standard 60-min incubation. Each term represents the mean ± SEM of five replicate experiments.

When LNL obtained from CFA-immunized animals (PPD-LNL) were added to Mφ monolayers which have been previously exposed to PPD (100 μg/ml), the degree of Mφ-LNL interaction observed after a standard 60-min incubation was not different than that observed when the same PPD-LNL were cultured with Mφ which had had no antecedent antigen exposure (Table I). Furthermore, in other experiments, when PPD (100 μg/ml) was added directly to the cultures and incubation of Mφ with PPD-LNL carried out for 1 h with PPD present continously in the medium, no difference in binding was seen compared to similar cultures without PPD. The binding of LNL from DNP-GPA or KLH immune animals by Mφ previously exposed in similar fashion to either DNP-GPA or KLH, respectively (100 μg/ml), likewise, was not different after a standard 1-h incubation than the binding of these LNL by Mφ which had not been previously exposed to antigen.

Effect of Prolongation of Culture on Macrophage-Lymphocyte Interaction. When PPD-LNL were cultured with glass-adherent Mφ for longer than 1 h (Fig. 1), there was a progressive decline in the number of LNL bound, such that after 20 h of incubation, binding was numerically equivalent to only 29% of binding seen after 1 h. On the other hand, when the Mφ had previously been exposed to PPD and PPD-LNL were added, this decrease in binding observed with time was not seen. By 8 h of culture there was a significant difference ($P < 0.001$) between the binding exhibited by Mφ previously pulsed with PPD and that of Mφ which had had no PPD exposure. Similar results were obtained when PPD (100 μg/ml) was added directly to the cultures and was thus present continuously through the period of incubation.

When PPD-LNL were themselves directly exposed to PPD (100 μg/ml × 37°C × 60 min), washed extensively, and then cultured for 20 h with Mφ which had had no antecedent antigen exposure, no significant degree of antigen-dependent binding developed. These PPD-pulsed LNL remained, however, capable of participating in antigen-mediated macrophage-lymphocyte interaction when cultured with PPD bearing Mφ.

The immune status of the animals from which the Mφ were obtained was not

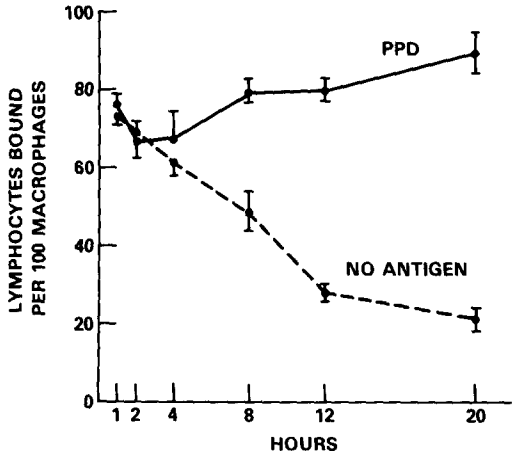

FIG. 1. The binding of LNL from PPD immune animals to syngeneic Mφ as a function of the length of time during which the two cell types were incubated together at 37°C. The Mφ had previously been exposed to either PPD (100 μg/ml) in PBS or PBS for 60 min at 37°C and washed four times before use. Each point represents the mean ± SEM of four replicate experiments.

found to be a significant variable as Mφ from unimmunized animals were observed to be as capable as those from CFA immunized animals at mediating antigen-dependent binding of PPD-LNL. The immune status of the animals from which the LNL were obtained was, however, found to be critical for the development of PPD-mediated macrophage-lymphocyte interaction. Adherence column-purified LNL were obtained from mesenteric lymph nodes of animals which had had no prior immunization procedures. These cells, when tested in a standard assay for the incorporation of tritiated thymidine (10), exhibited no proliferative response to varying doses of PPD, even when supplemental syngeneic Mφ were added, although they showed a marked proliferative response to phytohemagglutinin (Wellcome Research Laboratories, Beckenham, England). When binding was studied, it was observed that PPD (100 μg/ml)-pulsed Mφ bound no more of these LNL after a 20-h incubation than did Mφ unexposed to PPD.

The morphological features of macrophage-lymphocyte interaction observed after a 20-h incubation in the presence and absence of specific antigen are depicted in Fig. 2. In the absence of Mφ-bound antigen (Fig. 2 *a*) few LNL are seen. When Mφ bear specific antigen, clustering of LNL from immune animals develops (Fig. 2 *b*, *c*). The addition of 10×10^6 LNL resulted in a far greater number of LNL bound (Fig. 2 *c*) than the addition of the 5×10^6 LNL routinely used (Fig. 2 *b*). The vast majority of LNL were found in direct contact with Mφ. Occasionally, LNL were found not to be in direct contact with Mφ, but rather with other LNL which were, in turn, Mφ bound. Only rarely were LNL seen to be bound directly to the glass slide.

Of considerable interest was the decline in the degree of macrophage-lymphocyte interaction observed when cultures were prolonged in the absence of antigen. This could result from either a loss of cell functional capability or

number during the period of incubation. In order to determine the functional capabilities of the remaining cells, those LNL not bound by Mφ after a 20-h incubation in the absence of antigen were collected (viability >95% by trypan blue exclusion), washed, suspended in fresh medium RPMI-1640 with 10% FBS and presented to fresh Mφ monolayers. After a standard 1-h incubation, the binding of these LNL was similar to that observed when an equal number of fresh LNL were presented to the same Mφ. Similarly, when the Mφ which had been

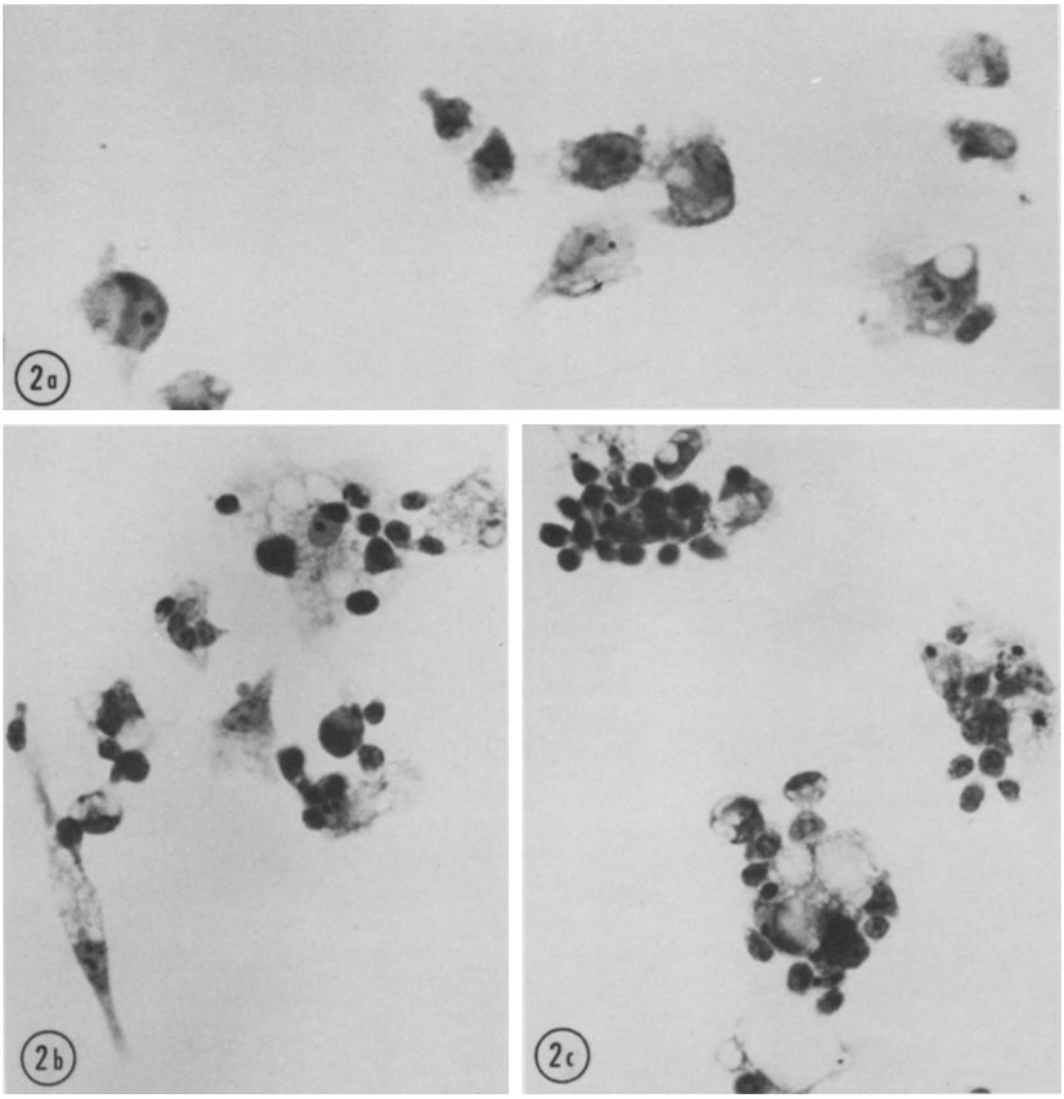

FIG. 2. Photomicrographs depicting the physical interaction between LNL from PPD immune guinea pigs and Mφ after a 20-h incubation. (*a*) Few LNL are bound by control Mφ which have had no antigen exposure. (*b*) Binding observed with the addition of 5×10^6 LNL to PPD bearing Mφ. (*c*) Binding exhibited when 10×10^6 LNL are incubated with PPD bearing Mφ.

cultured for 20 h with LNL were washed free of unbound cells and presented with fresh LNL, their ability to bind LNL was similar to control Mφ. Furthermore, we could not detect any activity in the supernates from 20-h old cultures of Mφ and LNL which, even undiluted, inhibited binding. Finally, when PPD was added to cultures of PPD-LNL and Mφ which had been incubated for 20 h in the absence of PPD (Fig. 3), a rapid increase in binding was observed to levels similar to those seen when PPD was present from the initiation of culture. Thus, Mφ and LNL would appear to have maintained the ability to participate in both antigen-independent as well as antigen-dependent Mφ-LNL interaction during the 20-h period of culture in the absence of antigen. However, a decrease in the number of LNL present in the cultures was shown to occur as incubation was prolonged.

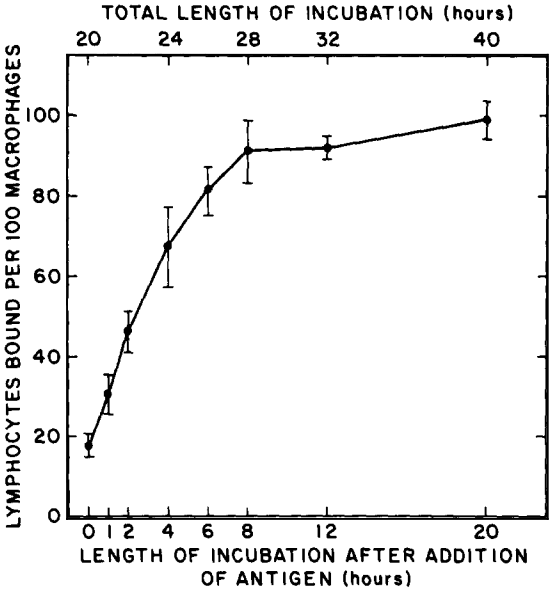

FIG. 3. The effect of adding PPD (100 μg/ml) to cultures of LNL from PPD immune animals and syngeneic Mφ which had previously been incubated for 20 h without PPD. Each point represents the mean ± SEM of three replicate experiments.

Although the LNL population were routinely more than 95% viable by trypan blue exclusion when cultures were initiated, only 40% or less of the LNL could be recovered after a 20-h incubation. This decrease in the number of LNL was observed in cultures containing either control or antigen-bearing Mφ and was far in excess of that which could be accounted for as bound by Mφ.

Effect of Antigen Concentration on Antigen-Dependent Macrophage-Lymphocyte Interaction. Mφ monolayers were exposed to varying concentrations of PPD for 60 min at 37°C and washed to remove excess antigen before the addition of LNL. The number of PPD-LNL bound by Mφ after a standard 20-h incubation was directly related to the log of the concentration of PPD to which the Mφ had been exposed (Fig. 4). Maximal binding resulted from Mφ exposure to a PPD concentration of 100 μg/ml. Mφ pulsed with this concentration bound 1.5–2% of the added PPD-LNL after a 20-h incubation.

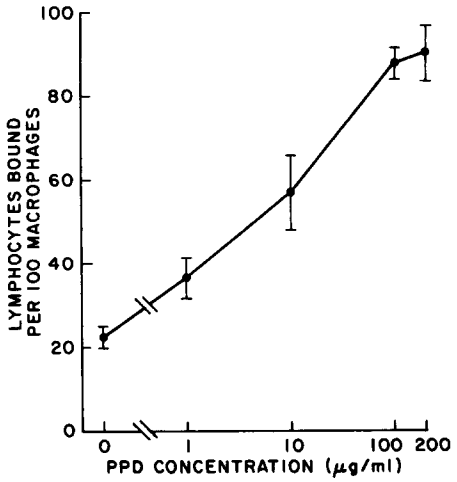

FIG. 4. The relationship between the concentration of the Mφ exposure to PPD and the subsequent binding of LNL from PPD immune animals observed after a standard 20-h incubation. Mφ were exposed to each concentration of PPD for 60 min at 37°C and washed four times before the addition of LNL. Each point represents the mean ± SEM of three or more replicate experiments.

Specificity of Antigen-Dependent Macrophage-Lymphocyte Interaction. For these studies, LNL were prepared from animals immunized with either CFA alone or DNP-GPA emulsified in CFA. The binding of each population of LNL by Mφ which had previously been exposed to either PPD (100 μg/ml), DNP-OVA (100 μg/ml), or two other dinitrophenyl conjugates, DNP-OVA or DNP-GL, was assessed after a standard 20-h incubation (Table II). PPD-pulsed Mφ bound about sixfold more PPD-LNL than Mφ which had had no previous antigen exposure, while Mφ exposed to any of the dinitrophenyl conjugates bound no more PPD-LNL than unpulsed Mφ controls. On the other hand, Mφ pulsed with either PPD (100 μg/ml) or DNP-GPA (100 μg/ml) bound markedly greater numbers of LNL obtained from DNP-GPA/CFA doubly immunized animals (DNP-GPA/PPD-LNL) than did Mφ which had not been exposed to antigen. However, Mφ exposed to either DNP-OVA (100 μg/ml) or DNP-GL (100 μg/ml) did not bind significantly different numbers of DNPGPA/PPD-LNL than did unpulsed Mφ. Furthermore, the addition of DNP-OVA (100 μg/ml) to cultures of DNPGPA/PPD-LNL and Mφ which had previously been expxed to DNP-GPA (100 μg/ml) did not interfere with the development of DNP-GPA antigen-specific Mφ-LNL interaction observed at 20 h.

Induction of DNA Synthesis in Lymphocytes Bound to Antigen-Bearing Macrophages. When incubation was continued beyond 20 h, a similar low level of Mφ-LNL interaction was observed for up to 72 h in the absence of a prior Mφ exposure to antigen (Fig. 5). However, Mφ which had been exposed to PPD before the addition of LNL exhibited markedly greater binding of PPD-LNL than Mφ without associated antigen for up to 72 h in culture. When incorporation of tritiated thymidine was evaluated autoradiographically, 9%, 32%, and 52% of the LNL bound by antigen-bearing Mφ were found to have taken up [^{3}H]TdR after 24, 48, and 72 h of culture, respectively (Fig. 5). Thymidine incorporation by

TABLE II

Specificity of Antigen-Dependent Physical Interaction Between Lymph Node Lymphocytes and Macrophages

Macrophage exposure to antigen*	LNL from guinea pigs immune to:	
	PPD	PPD + DNP GPA
	(LNL bound/100 macrophages‡)	
0	12.7 ± 2.9	15.3 ± 4.2
PPD	80.0 ± 10.9	101.7 ± 10.8
DNP-GPA	15.3 ± 4.8	103.8 ± 7.5
DNP-OVA	19.3 ± 4.5	22.1 ± 3.0
DNP-GL	19.3 ± 7.0	12.7 ± 1.6

* Established Mϕ monolayers were exposed to either PBS or one of the antigens in PBS, each at a final concn of 100 μg/ml, for 60 min at 37°C and washed before the addition of LNL.

‡ Data express LNL bound per 100 Mϕ after a standard 20-h incubation. Each term represents the mean ± SEM of three replicate experiments.

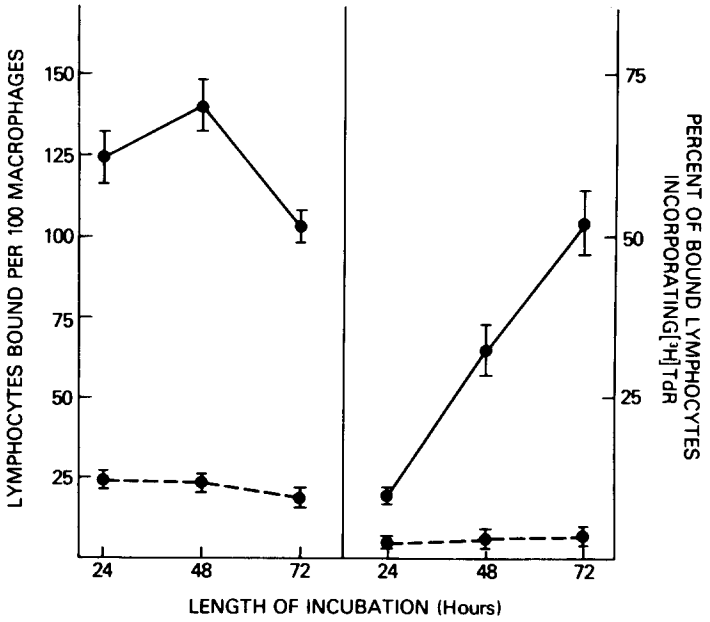

FIG. 5. The physical interaction between LNL from PPD immune guinea pigs and Mφ from syngeneic animals during prolonged incubation. The Mφ had previously been exposed to either PPD (100 μg/ml) in PBS or to PBS alone for 60 min at 37°C and washed four times before use. The points connected by the solid line (——) represent the binding mediated by PPD exposed Mφ while the dashed line (----) represents the binding mediated by control Mφ. The left panel indicates the number of cells bound per 100 Mφ while the right panel denotes the percent of Mφ bound LNL detected autoradiographically to be incorporating tritiated thymidine. Each point represents the mean ± SEM of three replicate experiments.

PPD-LNL found associated with control Mφ, which had not been exposed to PPD, remained at a constant low level (<4%) throughout the period of incubation.

Autoradiographs demonstrating the incorporation of tritiated thymidine by PPD-LNL bound to PPD-bearing Mφ after a 48-h incubation are depicted in Fig. 6. Of note is the observation that each of the LNL which has taken up [^{3}H]TdR is in direct physical contact with a Mφ.

The LNL which were not bound by Mφ were collected and similarly evaluated for the incorporation of tritiated thymidine. When unbound LNL were harvested from cultures containing no antigen and assayed autoradiographically, only a

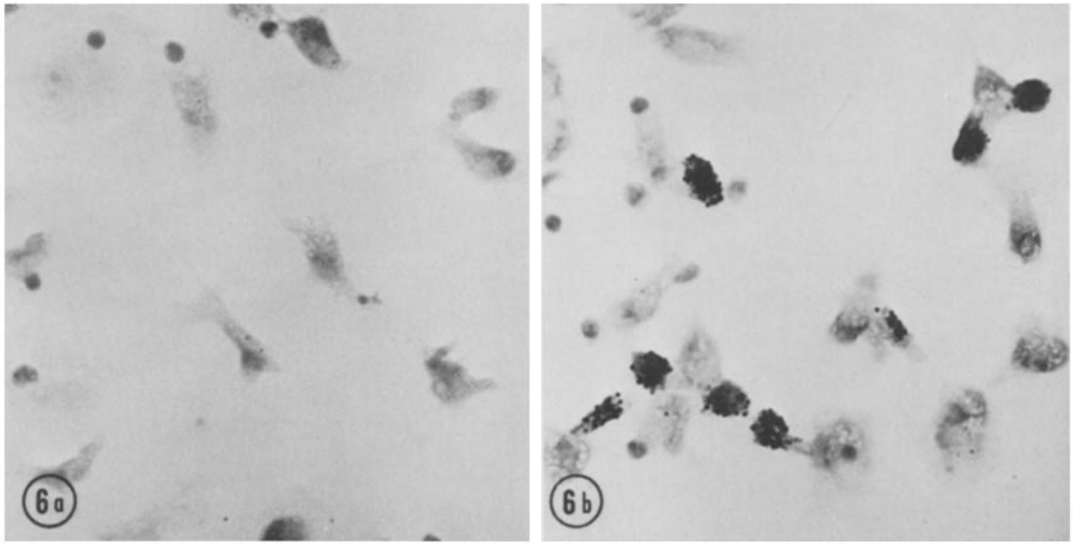

FIG. 6. Autoradiographs depicting the uptake of [^{3}H]TdR by LNL from PPD immune animals incubated for 48 h with (*a*) control Mφ and (*b*) PPD bearing Mφ. Although not all of LNL in the antigen-containing cultures are in direct contact with Mφ, the vast majority are. Each of the LNL which has incorporated [^{3}H]TdR is in direct contact with a Mφ. ×500.

small percent of them was found to have taken up [^{3}H]TdR after incubations of 24 h (1.1%), 48 h (0.9%), 72 h (3.0%), and 96 h (1.6%). When unbound PPD-LNL were collected from cultures containing PPD-bearing Mφ, a significant increase in the number which had taken up [^{3}H]TdR was apparent, but only after 72 h in culture. Thus, after 24, 48, 72, and 96 h incubations, 1.1%, 2.9%, 9.5% and 12.5% of the unbound LNL were found to have incorporated tritiated thymidine.

Antigen-Mediated Macrophage-Lymphocyte Interaction in Cultures Containing Both Antigen Exposed and Unexposed Macrophages. These experiments were designed to provide evidence as to whether immune lymphocytes, having encountered an antigen-bearing Mφ, remain physically associated with that particular Mφ throughout the period of blast transformation or, alternatively whether once triggered they are free to become associated with another, perhaps

nonantigen-bearing Mφ. For these experiments, Mφ monolayers were established from the same population of PEC on cover slips (9 × 20 mm). After overnight incubation, nonadherent cells were washed away. Each monolayer was then individually exposed to either antigen (PPD or KLH, final conc = 100 μg/ml) in PBS or PBS alone for 60 min at 37°C and washed. Antigen-pulsed and control monolayers were positioned side by side in the culture chambers such that added LNL would be free to interact with either or both populations of Mφ. LNL from appropriately immune animals were then added to the culture chambers and standard 20-h incubation on a rocking platform carried out. After incubation, the cover slips were individually washed free of unbound LNL and prepared as indicated in the methods for microscopic examination. The data presented in Table III indicate that antigen-mediated macrophage-lymphocyte

TABLE III

Antigen-Mediated Macrophage-Lymphocyte Interaction in Cultures Containing Both Antigen Exposed and Control Macrophages

Antigen exposure*		Lymph node lymphocytes bound per 100 macrophages‡	
Cover slip A	Cover slip B	Cover slip A	Cover slip B
–	–	17.3	18.3
+	–	85.0	19.5
–	+	16.5	91.5
+	+	86.0	82.5

* Mϕ monolayers, established on cover slips, were exposed to either antigen in PBS (either PPD or KLH, final concn = 100 μg/ml) or PBS alone for 60 min at 37°C. Monolayers were washed four times to remove antigen not associated with Mϕ. Cover slip monolayers were then placed side by side in culture chambers such that added LNL were free to interact with either population of Mϕ. LNL obtained from animals previously immunized to CFA and KLH were then added to the culture chambers.

‡ Data express the number of LNL bound per 100 Mϕ after a standard 20-h incubation. Each term represents the mean of two replicate experiments.

interaction involves only the antigen-bearing Mφ in cultures containing both antigen exposed and control unexposed Mφ. Furthermore, binding was found to be uniform throughout the control monolayer with no enhancement in the number of LNL bound by the Mφ on the edge of the cover slip directly adjacent to the antigen exposed cover slip monolayer. These data support the contention that when an immune LNL encounters an antigen-bearing Mφ, a prolonged interaction between the two cells results.

Histocompatibility Requirement for Antigen-Independent and Antigen-Mediated Macrophage-Lymphocyte Interaction. Previous studies have demonstrated that functional recognition of macrophage-associated antigen by immune thymus-derived guinea pig lymphocytes requires histocompatibility-linked identity between macrophage and lymphocyte (20). We therefore investigated the

role of histocompatibility in the development of physical interactions between macrophages and lymphocytes, both in the presence and absence of antigen.

Mφ monolayers were established by culturing PEC from either strain 2 or strain 13 unimmunized guinea pigs. Each of these populations of glass-adherent Mφ was then tested for its ability to bind LNL obtained from either strain 2 or strain 13 animals after the standard 60-min incubation used to evaluate antigen-independent macrophage-lymphocyte interaction (Table IV). There were no significant differences noted in the ability of either strain of Mφ to bind syngeneic or allogeneic LNL in the absence of antigen. Furthermore, there was no difference noted in the degree of endocytosis of LNL in the syngeneic or allogeneic combinations. Further experiments were performed using alloantisera supplied by Dr. E. Shevach, NIH (21). When binding was studied using allogeneic mixtures of LNL and Mφ, alloantisera directed against histocompatibility determinants on the Mφ did not decrease the binding of LNL when the

TABLE IV

Antigen-Independent Macrophage Binding of Syngeneic and Allogeneic Lymph Node Lymphocytes

Strain		Lymph node lymphocytes bound per 100 macrophages*
Macrophage	LNL	
2	2	74.7 ± 5.7
2	13	66.2 ± 5.3
13	13	70.3 ± 3.3
13	2	76.9 ± 7.8

* Data express the binding observed when 5×10^6 LNL from either strain 2 or 13 guinea pigs are presented to Mφ monolayers obtained from either strain 2 or 13 animals and incubated for 60 min in standard assay of antigen-independent macrophage-lymphocyte interaction. Each term represents the mean ± SEM of three or more replicate experiments.

antisera was present continuously during the incubation. Alloantisera directed against determinants on the LNL could not be evaluated because their presence in the incubation mixtures resulted in opsonization and phagocytosis of the LNL. Macrophage-thymocyte interaction could be studied, however, since alloantisera-mediated phagocytosis of thymocytes did not occur to a significant degree. Macrophage binding of thymocytes was not affected by the presence of alloantisera directed against determinants on either Mφ or thymocyte or both.

The development of antigen-mediated macrophage-lymphocyte interaction in syngeneic and allogeneic combinations of cells was next compared. Standard Mφ monolayers obtained from either strain 2 or strain 13 unimmunized animals were briefly exposed to either PPD (100 μg/ml) in PBS or PBS alone as indicated above. LNL obtained from either strain 2 or strain 13 guinea pigs which had been immunized with the same preparation of CFA were then added to each of these monolayers and standard 20-h incubation carried out. In the absence of antigen,

the same degree of interaction was observed in the syngeneic and allogeneic combinations (Table V), with no difference in the degree of endocytosis noted. In the cultures containing antigen-bearing Mφ, enhanced binding developed only when macrophages and lymphocytes were syngeneic. Furthermore, the addition of allogeneic PPD-LNL to cultures of PPD-LNL and syngeneic antigen-bearing Mφ did not inhibit the degree of syngeneic antigen-mediated interaction observed.

Discussion

Antigen-mediated in vitro proliferation of immune guinea pig lymph node lymphocytes is thought to involve the obligatory participation of macrophages (10). In this system, macrophages are thought to be the cells which must initially

TABLE V

Antigen-Mediated Macrophage-Lymphocyte Interaction: Histocompatibility Requirement

Strain		Macrophage exposure*	
Macrophage	LNL	Control	Antigen
		(LNL bound/100 macrophages)‡	
2	2	19.0 ± 5.8	111.9 ± 11.7
2	13	18.4 ± 6.7	25.1 ± 6.8
13	13	17.2 ± 3.5	99.5 ± 10.7
13	2	21.3 ± 4.6	33.0 ± 5.9

* Established Mϕ monolayers were exposed to either PPD (100 μg/ml) in PBS or PBS alone for 60 min at 37°C and washed four times before use.

‡ Data express the binding observed when 5×10^6 LNL obtained from either strain 2 or 13 PPD immune guinea pigs are added to antigen exposed or control Mϕ monolayers from strain 2 or 13 animals and incubated in standard fashion for 20 h. Each term represents the mean ± SEM of four replicate experiments.

interact with antigen in order to facilitate its functionally effective presentation to immune lymphocytes. Macrophages, briefly exposed to antigen before mixing with lymphocytes, have been demonstrated to take up sufficient antigen for efficient lymphocyte triggering (10, 22). The present studies were undertaken to more precisely analyze the sequence of physical events involved in immune lymphocyte activation. Thus, a modified antigen pulse technique was utilized, whereby glass-adherent Mφ were briefly exposed to various soluble antigens and washed before the addition of immune lymphocytes. When tritiated DNP-GPA (prepared from 2-4 dinitrofluorobenzene[3,5^{3}H] and guinea pig albumin) was used as an antigen, such exposure resulted in autoradiographically detectable uptake by more than 90% of the Mφ. Similarly, a standard exposure to horseradish peroxidase (HRP, Worthington Biochemical Corp., Freehold, N. J.) or KLH coupled to HRP, each at 100 μg/ml, resulted in uptake by more than 95% of Mφ when assessed by the cytochemical development of HRP reaction product (23). No significant amount of glass-bound antigen was found utilizing either of

the aforementioned techniques.[2] Moreover, antigen carry over could be evaluated in the cultures of antigen-bearing Mφ by mixing cell-free supernates of these cultures with immune peritoneal exudate lymphocytes, a highly antigen responsive lymphocyte population, and assaying for induction of [^{3}H]TdR incorporation (24). Using this technique, the amount of noncell-bound antigen present routinely in cultures after a standard Mφ exposure to antigen was found to be functionally equivalent to $\sim 1 \times 10^{-3}$ μg/culture.[2] Thus the technique of Mφ exposure employed resulted in uptake of antigen by the vast majority of Mφ with insufficient noncell-bound carry over to account for the degree of Mφ-LNL interaction which subsequently developed.

Antigen-mediated macrophage-lymphocyte interaction develops when LNL obtained from animals immunized to a particular antigen are incubated with macrophages which have had previous exposure to that antigen. This phenomenon requires (*a*) prolonged incubation to develop, (*b*) the coincidence in culture of macrophages bearing specific antigen with lymphocytes obtained from animals immune to that antigen, and (*c*) the sharing of histocompatibility-linked determinants by macrophage and lymphocyte. Moreover, antigen-dependent binding is not mediated solely through hapten specificity when hapten-protein conjugates are used, but, appears to be principally carrier- or conjugate-specific. The observation that similar degrees of antigen-mediated macrophage-lymphocyte interaction developed when antigen was present continuously in the medium indicates that such interactions result not because antigen is physically limited to the Mφ but, rather suggests that these approximations represent an obligatory event in the triggering of immune lymphocytes.

The functional significance of antigen-mediated macrophage-lymphocyte interactions is indicated by the observation that tritiated thymidine incorporation is initially manifested only by those lymphocytes clustered about antigen-bearing Mφ and is apparent within 48 h in culture. Significant numbers of unbound lymphocytes are observed to have incorporated [^{3}H]TdR only after 72 h of incubation supporting the contention that activation of the lymphocytes is initiated while they are in physical contact with the Mφ. In order to determine if lymphocyte DNA synthesis is necessary for the development of antigen-mediated macrophage-lymphocyte interaction, experiments were done using mitomycin-C-treated immune LNL. While these cells exhibited no proliferative response to Mφ-bound antigen in standard assays of [^{3}H]TdR incorporation, they were bound by antigen-bearing Mφ in similar fashion as untreated LNL indicating that the development of antigen-mediated macrophage-lymphocyte interaction does not depend on the induction of lymphocyte DNA synthesis.

These observations, as well as those of others (13, 25), indicate that physical interaction between macrophages and lymphocytes plays an important role in the development of antigen-mediated proliferation of immune lymphocytes. The studies reported here extend this concept to imply that the physical events involved in lymphocyte activation proceed sequentially from antigen-independent reversible binding of lymphocytes by Mφ to antigen stabilized specific interaction. Antigen-independent binding may initially and, reversibly, bring lymphocytes into close anatomic association with Mφ. When Mφ have taken up

[2] Lipsky, P. E., and A. S. Rosenthal. Unpublished observations.

and retained specific antigen, this interaction could facilitate the efficient presentation of antigen to committed lymphocytes. Transmission of an antigenic signal would then result in prolonged macrophage-lymphocyte interaction eventuating in lymphocyte proliferation and/or differentiation. Such an interpretation is supported not only by the findings presented in these studies but also by the microcinematographic demonstrations (26, 27) of the prolonged clustering of lymphocytes and blast cells about Mφ, presumably bearing an antigenic stimulus.

The histocompatibility requirements for antigen-independent and antigen-mediated macrophage-lymphocyte interaction differ. Total identity at histocompatibility loci is not required for antigen-independent binding of lymphocytes by Mφ. Since strain 2 and 13 guinea pigs have been demonstrated, however, to share a major histocompatibility antigen (28), it remains possible that Mφ-LNL interaction could require identity at such a shared locus and not at the loci which were disparate in the animals studied. The development of antigen-mediated macrophage-lymphocyte interaction, on the other hand, requires that macrophages and lymphocytes share histocompatibility-linked determinants. These findings are consonant with the observation that the functional recognition of soluble protein antigens by guinea pig thymus-derived lymphocytes requires the presentation of antigen by histocompatible Mφ (20). Furthermore, these data support the concept that functional activation of immune thymus-derived lymphocytes by antigen-bearing macrophages requires physical interaction which is mediated by membrane products coded for by genes of the histocompatibility region or genes linked to them.

The mechanism by which the presence of antigen in macrophages results in prolonged macrophage-lymphocyte interaction has not been precisely defined. The experiments utilizing two separate monolayers in the same culture chamber, one bearing antigen and the other not, indicate that antigen-mediated binding predominantly involves those Mφ actually bearing antigen. The observation that control Mφ exhibit no enhanced binding in these experiments further suggests that antigen-mediated interaction is a primary event rather than a secondary manifestation of lymphocyte activation. The data indicating that antigen-mediated binding precedes the induction of lymphocyte DNA synthesis support this interpretation. Thus, it is likely that antigen-mediated macrophage-lymphocyte interaction represents an early step in the sequence of events leading to antigen recognition.

Summary

The effect of specific antigen on the development of physical interactions between lymph node lymphocytes (LNL) obtained from animals which had been immunized to that antigen and macrophages was examined. We found that the presence of antigen, either limited to the macrophage (Mφ) or free in the medium, profoundly increased the degree of Mφ-LNL interaction observed. This enhanced interaction was dependent on the coincidence in the cultures of Mφ bearing antigen and LNL from animals specifically immunized to that

antigen. Although antigen-independent interactions developed equally well between syngeneic and allogeneic combinations of lymphocytes and macrophages, antigen mediated interactions required that macrophages and lymphocytes be syngeneic. Prolongation of antigen-mediated Mφ-LNL interactions resulted in the induction of LNL DNA synthesis, initially involving those lymphocytes physically associated with antigen-bearing Mφ. These studies are interpreted to indicate that physical interaction between immune lymphocytes and antigen-bearing Mφ represents a morphological correlate of the functional activation of immune lymphocytes. Further, it is suggested that the physical events involved in lymphocyte proliferation may proceed sequentially from antigen-independent reversible binding of lymphocytes by macrophages to prolonged antigen-stabilized interaction eventuating in the triggering of specifically immune lymphocytes.

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