

MACROPHAGE-LYMPHOCYTE CLUSTERS IN THE IMMUNE RESPONSE TO SOLUBLE PROTEIN ANTIGEN IN VITRO

I. Roles of Lymphocytes and Macrophages in Cluster Formation

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Studies from recent years have provided a vast body of data concerning in vitro cell-mediated immune reactions toward soluble protein antigens. A few hours after exposure of immune lymphoid cells to antigen a population of lymphocytes start releasing mediators, such as migration inhibitory factor (MIF)¹ (1-4) and chemotactic factor (5), and upon continued culture a population of lymphocytes initiate blast transformation and cell division (6, 7). Although it is clear that these phenomena are due to stimulation of antigen-specific lymphocytes by antigen, the nature of the stimulatory signal is unknown. It is widely assumed that an early step in the stimulation that leads to blast transformation is binding of antigen to T-lymphocyte receptors. However, T lymphocytes do not bind easily detectable amounts of antigen to their surface (8) and pure populations of lymphocytes display a poor proliferative response to antigen (9-12), indicating a role for an accessory, antigen-binding cell in the generation of the stimulatory signal. Several studies have conclusively demonstrated that macrophages can serve in that role. Thus, addition of macrophages pulsed with purified protein derivative of tuberculin (PPD) by brief exposure in vitro induces blast transformation in purified populations of syngeneic immune lymphocytes from guinea pig (11-14), rabbit (9, 15), and man (16, 17). Some data suggest that this macrophage-lymphocyte cooperation requires physical interaction between the two cells (9, 15, 16) during which the macrophage presents molecules of surface-bound antigen to the lymphocyte (14-18).

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¹ *Abbreviations used in this paper:* BSS, balanced salt solution; DT, diphtheria toxoid; FCS, fetal calf serum; HRPO, horse radish peroxidase; LNC, lymph node cells; LNL, adherence column-passed LNC; MEM-FCS, completely supplemented Eagle's minimal essential medium containing 15% heat-inactivated FCS; MIF, migration inhibitory factor; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; PEM, peritoneal exudate macrophages; PPD, purified protein derivative of tuberculin.

In the present paper we want to focus upon physical interaction between guinea pig macrophages and lymphocytes during the immune response in vitro. This has been studied previously by Salvin (19, 20) and by Seeger and Oppenheim (11) using lymph node cells as the source of lymphocytes and syngeneic peritoneal exudate cells as the source of macrophages. During culture of cells from immune animals with PPD, they observed, lymphocytes and macrophages tended to become closely associated, with lymphocytes clustering around individual macrophages. If the role of macrophages in antigen-induced lymphocyte proliferation is presentation of surface-bound antigen to lymphocytes, clustering of lymphocytes around macrophages would appear to be a suitable arrangement for this presentation. We have studied the formation of macrophage-lymphocyte clusters during the early in vitro response to antigen of mixtures of peritoneal macrophages and lymph node lymphocytes from guinea pigs immunized with tubercle bacilli. We report that the formation of cell clusters is antigen-specific, with the lymphocytes acting as specific cells and the macrophages as antigen-binding (or -processing) cells.

Methods

Animals. Inbred and random bred male and female guinea pigs, strain Ssc:AL, weighing 400–500 g from Statens Seruminstitut, Copenhagen, were used.

Immunization. Guinea pigs were immunized against tubercle bacilli by intracutaneous injections in four sites of the abdominal skin of 0.1 ml of a suspension of dried heat-killed *Mycobacterium tuberculosis*, containing 40 μg /0.1 ml in Marcol paraffin oil (Tuberculin Department, Statens Seruminstitut, Copenhagen). 2–4 wk later the immunized guinea pigs were skin-tested by double intracutaneous injections of PPD (Tuberculin Department, Statens Seruminstitut, Copenhagen) containing 2 μg /0.1 ml phosphate buffered saline, pH 7, 4 (PBS). Guinea pigs were immunized with horse radish peroxidase (HRPO, Sigma Corporation, St. Louis, Mo.) or with diphtheria toxoid (DT, Statens Seruminstitut, Copenhagen) by intracutaneous injections in four sites of the abdominal skin of 0.1 ml of an emulsion made by mixing HRPO in PBS, 2.5 mg/ml or DT in PBS, 6.0 mg/ml with an even volume of complete adjuvant (Difco Laboratories, Detroit, Mich.). 2–4 wk later the immunized guinea pigs were skin-tested by intracutaneous injections of 0.5, 5, and 50 μg of the antigen in 0.1 ml PBS. Guinea pigs with positive 48-h skin tests were used within 8 wk after testing.

Cell Suspension. To obtain peritoneal exudates guinea pigs were injected intraperitoneally with 10 ml sterile paraffin oil. Exudates were harvested 3 days later by intraperitoneal injection of 10–20 ml cold balanced salt solution (BSS). Axillary and inguinal lymph nodes were removed and pooled in cold BSS. The lymph nodes were cut with scissors and squeezed through a no. 100 stainless steel mesh with BSS to make a single cell suspension. The suspensions of lymph node cells (LNC) and peritoneal exudate cells (PEC) were washed in BSS twice by centrifugation at 175 g for 7 min at room temperature. The cells were resuspended in completely supplemented Eagle's minimal essential medium containing 15% heat-inactivated fetal calf serum (MEM-FCS).

Purification of LNC on Adherence Column. Adherence columns (12) that retain macrophages, neutrophils, and B lymphocytes (but allow passage of T lymphocytes) were prepared in 50-ml glass syringes with a 2 cm bottom layer of perlon wool, 30 ml of washed acid-cleaned glass beads, 80–100 μm in diameter, and a 2 cm top layer of perlon wool. The column was allowed to equilibrate to 37°C for 60 min and was rinsed with 37°C MEM-FCS immediately before use. 100–200 $\times 10^6$ LNC were suspended in 5 ml of pure fetal calf serum (FCS) inactivated at 56°C for 30 min. The cell suspension was poured onto the column and incubated at 37°C for 30 min. Nonadherent LNC were eluted at 2 ml/min with 50 ml of 37°C MEM-FCS. The number of eluted cells was about 25% of the number added to the column. More than 97% of the column-passed cells (LNL) excluded trypan blue.

Cell Culture. In the standard experiment monolayers of peritoneal exudate macrophages (PEM) were established by culture of 0.4 ml PEC-suspension containing 1.6×10^6 cells in each of the

compartments of Lab-Tek tissue culture chamber slides (Lab-Tek Products, Division Miles Laboratories Inc., Naperville, Ill.). After culture of the cells for 120 min at 37°C in an atmosphere of 5% CO₂ in air nonadherent cells were removed by flushing the culture chambers four times with 37°C MEM-FCS. That the cells remaining on the slide floor of the culture chamber were actually macrophages was verified by exposing the cells of occasional cultures to latex particles (see below). More than 99% of the cells took up latex particles during a 60 min culture period at 37°C.

4×10^5 LNL were added to the macrophage monolayer in each of the compartments of the culture chamber in a vol of 0.4 ml MEM-FCS to which antigen (or an equivalent volume of PBS) had been added to a concentration of 10 µg/ml. The cells were cultured in quadruplicate or triplicate on a stationary platform for 20 h at 37°C in an atmosphere of 5% CO₂ in air. Cultures were terminated while remaining in the incubator by the injection into each of the culture chamber's compartments of 0.1 ml of a 7.5% solution of glutaraldehyde in 0.1 M cacodylate buffer. After 30 min of fixation the compartments were rinsed in distilled water and the culture chamber's plastic superstructure was removed. The slide base of the culture chamber with adherent cells was now ready for histologic processing.

Technique for Brief Exposure of Cells to PPD. PEM were briefly exposed to PPD by culture of the cells for 120 min with MEM-FCS containing PPD 20 µg/ml in Lab-Tek tissue culture chambers at a cell concentration of 1.6×10^5 cells/ml followed by flushing of the culture chambers four times with 37°C MEM-FCS. LNL were briefly exposed to PPD by culture of the cells for 120 min with MEM-FCS containing PPD 20 µg/ml in 40 ml Falcon tissue culture flasks (Falcon Plastics, Div. of BioQuest Oxnard, Calif.) at a cell concentration of 4×10^5 cells/ml. The cells were washed in MEM-FCS by centrifugation three times at 175 g for 7 min at room temperature.

Technique for Latex-Labeling of Macrophages. 5 ml of a suspension containing 5×10^6 PEC in MEM-FCS was mixed with 0.1 ml of Bacto-latex \times 0.81 latex beads (Difco Laboratories, Detroit, Mich.) in BSS and added to 40 ml Falcon culture flasks in 5-ml vol. After culture at 37°C for 60 min in an atmosphere of 5% CO₂ in air, nonadherent cells and free latex beads were removed by flushing the culture chambers four times with 37°C MEM-FCS.

Production of Supernates from Cultures of LNC and Testing for Migration Inhibitory Factor. Details of the agarose plate technique will be described in a separate communication.² Briefly, to suspensions containing 3.6×10^6 LNC plus 0.4×10^6 PEC/ml and to suspensions containing 24×10^6 LNC/ml was added either PPD to a concentration of 50 µg/ml or an equivalent vol of PBS. The cells were cultured either in 40-ml Falcon tissue culture flasks or in 12 \times 100 mm plastic tubes for 24 h at 37°C in 5% CO₂ in air. After 24 h the cells were spun down at 600 g for 10 min, the supernates were drawn and kept at -20°C until testing. Supernates were tested for inhibitory activity against normal guinea pig PEC by suspending these in supernate at a concentration of 80×10^6 cells/ml. The cells were preincubated for 90 min at 37°C in 5% CO₂ in air and were then resuspended. 7-µl portions of the cell suspension was deposited in the holes (2.3 mm in diameter and 4 mm in height) of agarose layers in plastic Petri dishes and incubated at 37°C for 24 h in a moist atmosphere of 5% CO₂ in air. At the end of the incubation period the cells were fixed with glutaraldehyde and migration areas were measured by planimetry.

Microscopic Study of Cultures. The adherent cells on the slide base of the culture chambers were stained with the May-Grünwald-Giemsa stain and a cover slip was applied. The macrophage-lymphocyte clusters in the cultures were counted in a Leitz Orthoplan microscope at 100 times magnification using an ocular grid to screen the entire 1 cm² area of the slide, which formed the base of a culture compartment. The extent to which the count reflects the true number of clusters produced by the live cells depends upon the preservation of already established clusters during fixation of cultures. Comparison of identical fields of cluster-containing cultures before and after fixation showed no difference in numbers of clusters.

Results

Light Microscopic Observations of Macrophage-Lymphocyte Clusters. In most cultures the macrophage monolayer was of fairly even density throughout

²Brændstrup, O., M. Magnusson, J. Maxild, and O. Werdelin. Manuscript submitted for publication.

on the culture chamber slide base. The lymphocytes, on the other hand, were never evenly distributed, but were concentrated in a corner or along one margin of the culture chamber. Macrophages and lymphocytes were easily distinguished in Giemsa-stained slides on the basis of morphology, size, and staining. As expected from preliminary experiments macrophages and lymphocytes from immune guinea pigs formed clusters during culture with specific antigen.

To evaluate the difference with respect to cluster formation by immune and nonimmune cells, counts were made of the number of lymphocytes attached to each of 1,000 macrophages in a culture of immune and in a culture of nonimmune cells with PPD (Fig. 1). For analysis was chosen the area in the culture chamber in which the lymphocytes were located in the highest numbers. In both cultures the majority of the macrophages were associated with none or a few lymphocytes. The striking difference between the two cultures was the number of macrophages with seven or more lymphocytes attached. Thus, in the culture of immune cells 167 out of 1,000 macrophages had seven or more lymphocytes attached, while in

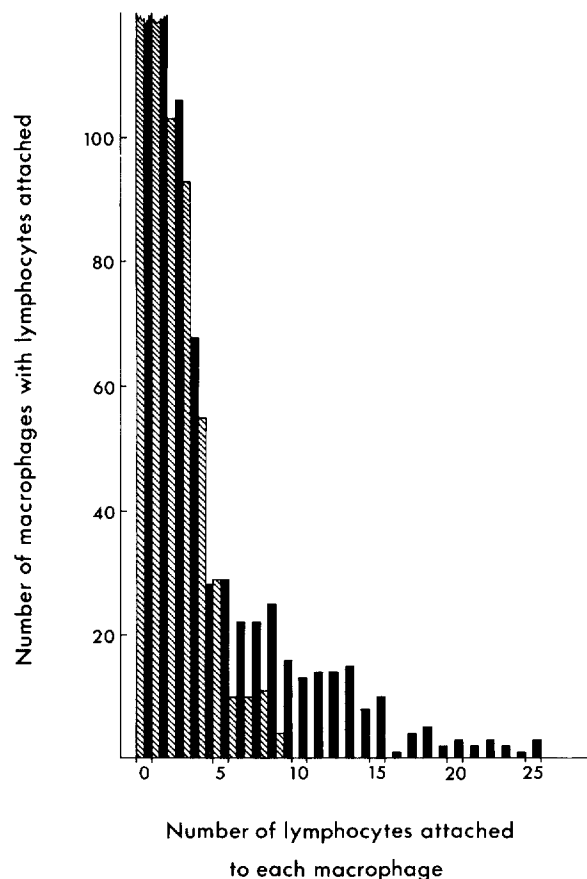


FIG. 1. Histogram showing the number of lymphocytes attached to each of 1,000 macrophages in a culture of immune cells (black columns) and in a culture of nonimmune cells (hatched columns) with PPD.

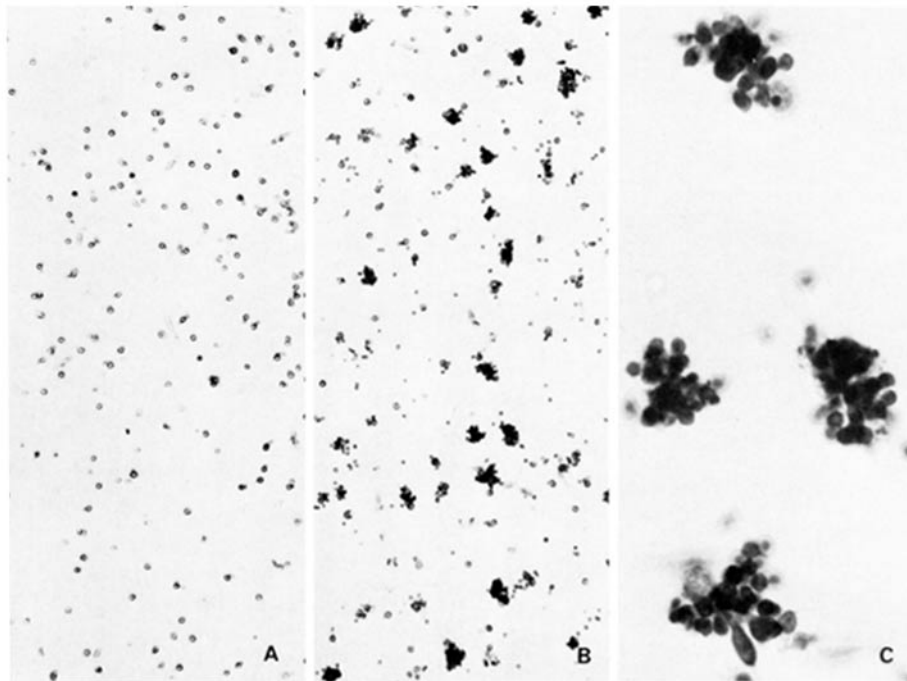


FIG. 2. Macrophage-lymphocyte clusters in 20-h cultures of 4×10^5 column-purified lymph node cells on monolayers of autologous macrophages. (A) Nonimmune cells cultured with PPD $10 \mu\text{g/ml}$. Very few lymphocytes are attached to the macrophages. $\times 100$. (B) Immune cells cultured with PPD $10 \mu\text{g/ml}$. Many lymphocytes are clustering around some of the macrophages. $\times 100$. (C) Detail of B. $\times 525$.

the culture of nonimmune cells only 20 out of 1,000 macrophages had. Fig. 2 *a* and *b* show representative areas from the two cultures. The cell clusters typically contained one macrophage and several lymphocytes which appeared as if they were sprouting out from the macrophage in a bunchlike pattern (Fig. 2 *c*). It was decided, for the purpose of counting, to reckon as macrophage-lymphocyte clusters only aggregates of cells in which seven or more lymphocytes were attached to a macrophage.

The Influence of Macrophage Monolayer Density on the Number of Clusters Produced by Immune Cells. Culture chambers with macrophage monolayers of varying densities were established and 4×10^5 immune autologous LNL were added to each chamber. The mean number of clusters produced per culture was roughly the same over the range of macrophage densities produced by adding from 3.2 to 0.4×10^5 PEC to the culture chambers (Fig. 3). At lower densities the number of clusters was lower. In subsequent experiments macrophage monolayers were produced by adding 1.6×10^5 PEC to each culture chamber.

Cluster Formation by LNC Enriched in T Lymphocytes. The number of clusters produced by 4×10^5 LNL and by 4×10^5 not-column-purified LNC on macrophage monolayers were compared. LNL produced a mean of 1217 clusters per culture, while not-column-purified LNC from the same guinea pig produced a

mean of 684 clusters per culture (Table I). In all subsequent experiments we used 4×10^5 LNL for incubation on the macrophage monolayer.

Time and Dose Dependency of Macrophage-Lymphocyte Cluster formation. Replicate cultures of cells from a guinea pig sensitized to *M. tuberculosis* were grown with PPD and terminated after 2, 8, 14, and 20 h. (Fig. 4). By 2 h cultures of immune cells had formed 0 clusters, and by 8 h a mean of 891. The number of clusters per culture did not further increase by 14 or 20 h.

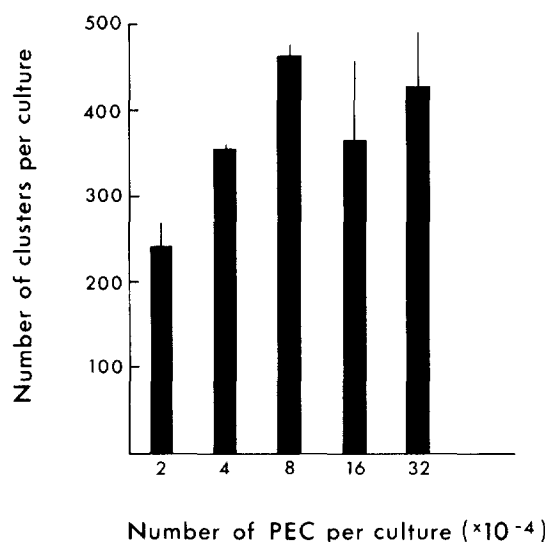


FIG. 3. Number of clusters produced in cultures of immune cells with PPD in the medium at varying densities of macrophage monolayers (numbers indicate the numbers of PEC used for establishing macrophage monolayers). Mean of three or four cultures. Vertical bars indicate SD.

TABLE I
Effect of Column-purification of Lymph Node Cells on the Formation of Clusters in Cultures Containing Antigen

Pre-treatment of lymph node cells*	No. of clusters per culture‡
Column-purification	1,217 \pm 203
None	684 \pm 49

* LNC from a guinea pig immunized with *M. Tuberculosis* were or were not passed through an adherence column and then incubated with PPD 10 μ g/ml on monolayers of autologous PEM.

‡ Mean of three or four cultures \pm SD.

Cells from a tuberculin-sensitized guinea pig were cultured for 20 h with various concentrations of PPD from 0.001 to 50 μ g per ml (Fig. 5). Increasing numbers of clusters were generated by culture with increasing concentrations of PPD from 0.01 μ g per ml medium up to the concentration 10 μ g per ml. Further increase of the PPD-concentration to 20 or 50 μ g per ml did not result in increase of the number of clusters.

Specificity of Macrophage-Lymphocyte Cluster Formation. Experiments

were carried out to investigate if cluster formation was antigen-specific. Cells from guinea pigs sensitized either to HRPO or to DT, or deriving from nonimmune guinea pigs were tested for their ability to form cell clusters in culture with HRPO and DT. Immune cells formed significant numbers of cell clusters only with the antigen to which the animal had been sensitized (Table II). Nonimmune cells formed very few clusters with either of the antigens.

The finding that the formation of macrophage-lymphocyte clusters was antigen-specific prompted experiments to determine if the cells in which the specificity resides were among the LNL or among the PEM. Immune LNL (from guinea pigs immunized with *M. tuberculosis*) were incubated on monolayers of either autologous PEM or syngeneic nonimmune PEM, and nonimmune LNL were incubated on monolayers of either autologous PEM or syngeneic immune PEM. The cells were cultured with PPD and the resulting numbers of

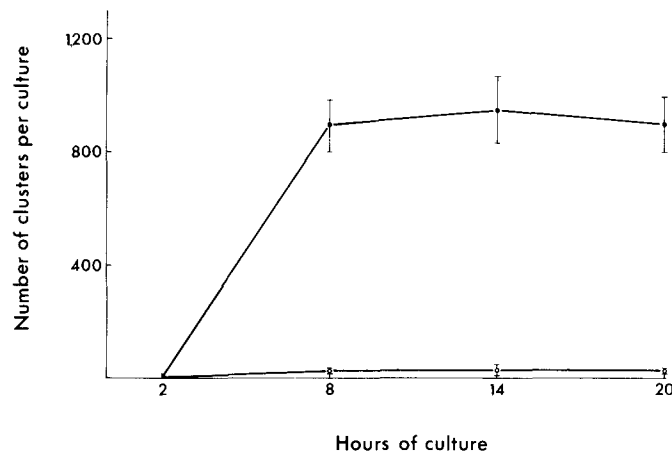


FIG. 4. Time sequence of cluster formation in cultures of immune cells with (●—●) and without (○—○) PPD in the medium. Mean of three or four cultures. Vertical bars indicate $2 \times$ SD.

macrophage-lymphocyte clusters were enumerated (Table III). Both combinations of cells containing immune LNL developed many macrophage-lymphocyte clusters, while those containing nonimmune LNL developed few. The experiment indicates that the formation of macrophage-lymphocyte clusters is caused by immune lymphocytes and that macrophages play a nonspecific role in the cluster formation.

The Effect of Brief Exposure of Immune Cells to Antigen on the Formation of Macrophage-Lymphocyte Clusters. Recent studies (9–12, 14), indicate that immune lymphocytes are unable to bind sufficient soluble antigen to allow for their subsequent antigen-induced proliferation *in vitro*. Instead, macrophages appear to be the antigen-binding cells which offer the antigen to the immune lymphocytes. Experiments were undertaken to see if this is the case also in antigen-induced macrophage-lymphocyte cluster formation. Monolayers of PEM and suspensions of LNL from an immune guinea pig were incubated with PPD *in vitro* for 120 min, then washed thoroughly, and combined with untreated cells of

the other type in appropriate numbers. The cells were then cultured for 20 h without fresh PPD added to the medium. Control cultures of untreated cells were grown with and without PPD added to the medium. The result (Table IV) shows that LNL incubated on monolayers of PPD-pulsed PEM formed macrophage-lymphocyte clusters in numbers comparable to the numbers in control cultures of the same cells with "free" PPD in the medium. On the other hand PPD-pulsed LNL incubated on monolayers of untreated PEM did form macrophage-lymphocyte clusters in numbers which were about one sixth of the numbers in control cultures of the same cells with free PPD in the medium, although in numbers significantly higher than control cultures of the same untreated cells.

To investigate if the clusters developing in cultures containing both PPD-

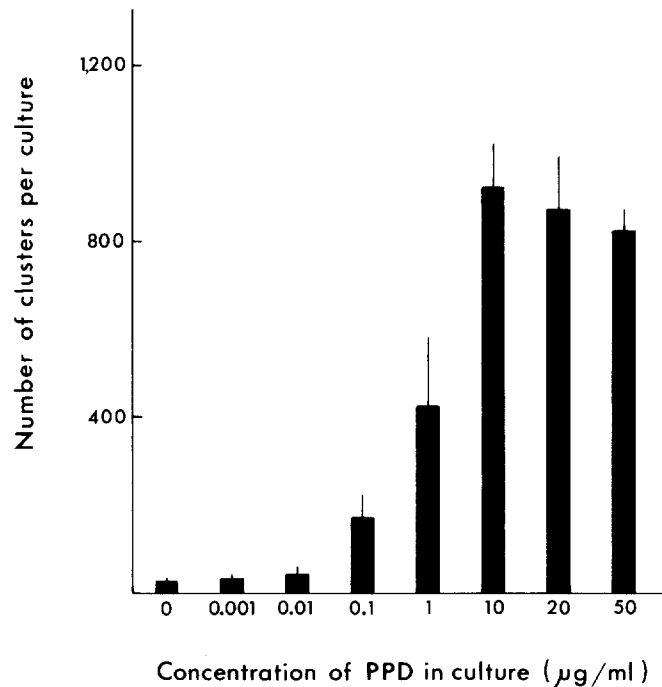


FIG. 5. Dose dependency of cluster formation. Mean of three or four cultures. Vertical bars indicate SD.

exposed and not-antigen-exposed macrophages were all associated with the PPD-exposed macrophages the following experiment was carried out. A mixed macrophage monolayer containing 1×10^6 latex-labeled, PPD-exposed PEM and 1×10^6 unlabeled, not-antigen-exposed PEM was established in a 40-ml Falcon culture flask, and 18×10^6 LNL were incubated for 20 h on the monolayer in the absence of free PPD. Microscopic examination revealed a tendency to formation of clusters containing more than one macrophage. 100 clusters—all containing only one macrophage—were selected for analysis. In 89 of these a latex-labeled macrophage could be identified with certainty (Table V). In a control culture with a mixed macrophage monolayer containing labeled, not-antigen-exposed PEM and unlabeled, PPD-exposed PEM a latex-labeled macrophage could be

identified in only 14 out of 100 clusters. The result indicates that in cultures with no free antigen the vast majority of clusters develop with macrophages which have been exposed to antigen.

The Effect of Supernates from Cultures of Immune Cells on the Formation of Macrophage-Lymphocyte Clusters by Normal Cells. Next, the possibility was tested that cluster formation is mediated by a product released from antigen-

TABLE II
Antigen-specificity of Cluster Formation

Cells from guinea immunized with:*	Antigen in culture medium	No. of clusters per culture‡
HRPO	HRPO	129 (107-154)
	DT	2 (0-3)
	None	2 (1-3)
DT	DT	74 (51-88)
	HRPO	4 (2-4)
	None	4 (3-5)
Nonimmune	HRPO	2 (0-2)
	DT	2 (0-4)
	None	3 (1-5)

* LNL from guinea pig immunized with the antigen in complete adjuvant were incubated on monolayers of autologous PEM.

‡ Mean of three or four cultures and range.

TABLE III
Production of Clusters by Combinations of Immune and Nonimmune Lymphocytes and Macrophages in Cultures Containing Antigen

Cell combination*	No. of clusters per culture‡
Immune macrophages + immune lymphocytes	1,659 ± 188
Immune macrophages + nonimmune lymphocytes	12 ± 5
Nonimmune macrophages + immune lymphocytes	1,876 ± 59
Nonimmune macrophages + nonimmune lymphocytes	10 ± 6

* Immune cells deriving from a guinea pig immunized with *M. tuberculosis* and nonimmune cells deriving from a syngeneic guinea pig were combined and cultured with PPD 10 µg/ml.

‡ Mean of three or four cultures ± SD.

stimulated immune lymphocytes into the culture medium. In one series of experiments mixtures containing 2×10^6 PEC and 18×10^6 LNC from an immune guinea pig were cultured in Falcon flasks with and without PPD added to the medium for 20 h in 5 ml of MEM-FCS. The supernates from these cultures were used for culturing nonimmune LNL on monolayers of autologous PEM. In two experiments the mean number of cell clusters per culture were 3(2-4) and 4(1-5), respectively, while the mean number of cell clusters formed by the same

nonimmune cells in control supernates (made by culture of the same immune cells without antigen) were 5(2-6) and 3(1-4), respectively. Although nonimmune cells did not form significantly higher numbers of macrophage-lymphocyte clusters in supernates from immune cells with antigen than they did in supernates from immune cells without antigen, the possibility remained that a cluster-inducing factor had been released from the immune lymphocytes during the initial culture, but was not present in the final supernates because it had been "consumed" by macrophages. In a second series of experiments immune LNC at a concentration of 24×10^6 LNC per ml without PEC added were

TABLE IV
Effect on Cluster Formation of Brief Pre-exposure to Antigen of Macrophages or Lymphocytes

Cells briefly exposed to antigen*	Free antigen in final culture	No. of clusters per culture‡
Macrophages	No	2,870 ± 68
Lymphocytes	No	530 ± 48
None	No	37 ± 12
None	Yes	2,946 ± 347

* Cells precultured with PPD 20 µg/ml for 2 h followed by washing of the cells three times in culture medium.

‡ Mean of three or four cultures ± SD.

TABLE V
Cluster Formation with Antigen-Pulsed and not-Antigen-pulsed Macrophages in Cultures with no Free Antigen

Macrophages labeled with latex	No. of clusters with a single:	
	Labeled macrophage	Unlabeled macrophage
Antigen-pulsed	89	11
Not antigen-pulsed	16	84

Macrophage monolayers were established by mixing approximately equal numbers of latex-labeled and unlabeled macrophages of which either the former or the latter had been pulsed with PPD, and syngeneic immune lymphocytes were incubated on monolayers.

cultured with PPD for 24 h. The supernates from two cultures of this type were tested in an indirect migration inhibition assay and were both found to be significantly inhibitory (per cent inhibition 31 and 42%, respectively). Nonimmune LNL cultured in these supernates on monolayers of autologous PEM produced a mean of 11(8-14) and 9(2-15) clusters per culture, respectively, compared to 4(0-8 and 4(2-8), respectively, in control supernates made by the same immune cells grown without antigen.

Relationship Between Numbers of Cells and Numbers of Macrophage-Lymphocyte Clusters Formed in Cultures. The failure to demonstrate a cluster-inducing effect of culture supernates of immune cells and antigen, and the demonstration that the vast majority of clusters developed with antigen-exposed

macrophages suggest that each cluster develops as the result of a direct physical interaction of one or a few immune lymphocytes with a macrophage. It was therefore of interest to see if the number of clusters developing in a culture is correlated to the number of immune cells present. LNL suspensions containing 1×10^6 cells per ml with 0, 25, 50, 75, and 100% immune cells were prepared by mixing LNL from a tuberculin-immune guinea pig with LNL from a syngeneic nonimmune guinea pig. The lymphocyte mixtures were cultured with PPD in the culture medium on monolayers of syngeneic nonimmune PEM. The number of clusters produced in the cultures (Fig. 6) was directly proportional with the number of immune LNL present.

Discussion

Macrophages and lymphocytes from guinea pigs immunized with *M. tuberculosis* form clusters of a unique structure during culture with tuberculin PPD (22). The clusters consist of a single macrophage adhering to the bottom of the culture vessel, and several lymphocytes, among which the central one is directly attached to the macrophage, while the remaining ones are all attached to the central one through their uropods. The present study outlines the operational specifications of cluster formation and defines the roles of macrophages and lymphocytes in the phenomenon.

Cluster formation was dose-dependent (Fig. 5). Over a range of antigen concentrations from 0.001 to 50 μg per ml, immune cells formed the highest

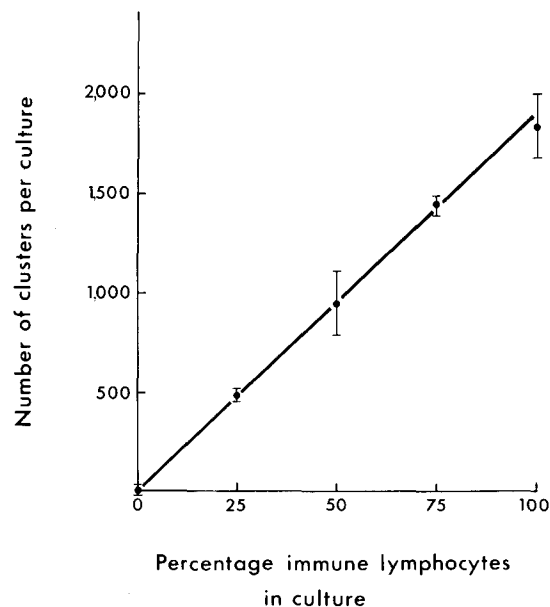


FIG. 6. Number of clusters produced in cultures of immune plus nonimmune lymphocytes varying in the percentage of immune cells on macrophage monolayers with PPD in the medium. Mean of three or four cultures. Vertical bars indicate $2 \times \text{SD}$.

number of clusters per culture with 10 μ g of PPD per ml culture medium. Cluster formation was time-dependent (Fig. 4). In cultures of immune cells with antigen, clusters were not found by 2 h, but were present in equal numbers by 8, 14, and 20 h. Cluster formation was antigen-specific. With the antigen used for immunization, LNL from guinea pigs immunized with either HRPO or DT produced a mean of 129 and 74 cells clusters per culture, respectively, on monolayers of autologous PEM. With no antigen or with the unrelated antigen, the same cells produced less than five cell clusters per culture (Table II). Previous reports by Salvin et al. (19, 20) have suggested that the antigen-specificity of cluster formation between macrophages and lymphocytes resides in the lymphocytes. This observation was confirmed in experiments in which immune LNL incubated with antigen on monolayers of nonimmune syngeneic PEM produced 1,700–1,800 clusters per culture, while nonimmune LNL incubated with antigen on monolayers of immune syngeneic PEM produced less than 20 per culture (Table III).

The role of the macrophages in cluster formation was explored in experiments in which lymphocytes were incubated on monolayers of peritoneal macrophages which had been briefly exposed to antigen and then washed repeatedly. Immune LNL cultured on monolayers of PPD-pulsed PEM produced the same number of clusters per culture as did the same cells cultured with free PPD (Table IV). On the other hand, PPD-pulsed immune LNL cultured on monolayers of untreated autologous PEM produced a number of clusters per culture which was only one sixth of the number produced by the same cells cultured with free PPD (Table IV). These data indicate that the macrophage of the cell cluster serves not merely as a passive recipient of the lymphocytes which are incorporated into the cell cluster, but as an active antigen-binding or -processing cell. A similar role as antigen-binding or -processing cell appears to be played by the macrophage in antigen-induced lymphocyte proliferation *in vitro*. A number of studies have shown that populations of immune lymphocytes deprived of glass-adherent cells respond poorly to PPD (9–12). The response is restored to optimum by the addition of syngeneic macrophages (11). Furthermore, macrophages which have been pulsed with PPD stimulate syngeneic immune lymphocytes just as effectively as macrophages plus free PPD in the concentration used for pulsing the macrophages (11–14). The mechanism by which antigen-pulsed macrophages stimulate immune lymphocytes to transformation is unknown. Several observations (9, 11–14, 16, 17) indicate that the macrophage-lymphocyte cooperation *in vitro* is not mediated by a factor released into the culture medium from the antigen-pulsed macrophages. Alternatively, the functional cooperation may require physical interaction between antigen-pulsed macrophage and immune lymphocyte.

The possibility was considered that cluster formation may be caused by a factor secreted by antigen-stimulated immune lymphocytes, similar to the macrophage aggregation factor described by Lolekha et al. (23). However, neither supernates of cultures of immune LNC plus PEC in which clusters had developed nor supernates of LNC with strong migration inhibitory activity did induce cluster formation in mixtures of nonimmune LNL and PEM. Our observation that the clusters produced by immune LNL on monolayers containing both antigen-exposed and not-antigen-exposed PEM, were nearly all associated with antigen-exposed macrophages (Table V), strongly supports the view that the

macrophage's contact with antigen, rather than with a lymphocyte-produced mediator, is a decisive event in cluster formation. We interpret these data to indicate that in the process of cell cluster formation macrophages serve as antigen-binding or -processing cells, while some immune lymphocytes interact physically and specifically with the macrophages.

If the immune lymphocytes interact physically and specifically with macrophages to produce cell clusters a quantitative correlation would be expected between the number of immune lymphocytes present and the number of clusters produced in a culture. The result of two experiments indicate that such a correlation exists. In the first, the number of clusters produced by incubation of a fixed number LNL with antigen on macrophage monolayers of varying densities was found to be the same over a wide of range macrophage monolayer densities (Fig. 3). In the second experiment the number of clusters produced by incubation of mixtures of immune and syngeneic nonimmune LNL with antigen on macrophage monolayers of the same density, was found to be directly proportional with the number of immune lymphocytes in the cultures (Fig. 6). Thus, the number of immune lymphocytes determines the number of clusters produced. Since only a fraction of the incubated lymphocytes are incorporated into clusters (4×10^5 LNL from guinea pigs immunized with *M. tuberculosis* produced from 400 to 3,000 clusters during culture with PPD, each containing a mean of approximately twelve lymphocytes) their formation must be due to the interaction of a subpopulation of lymphocytes with macrophages. Our ultrastructural study (22) has clearly established that only one of the cluster's lymphocytes interacts directly with the macrophage. Thus, presuming that each cluster is initiated by an interaction between a single lymphocyte and a macrophage, the size of the subpopulation can be estimated to be from approximately 0.1 to approximately 0.8% of the column-purified LNC.

The nature of the lymphocytes involved in cluster formation was partly characterized by the finding that LNC enriched in T lymphocytes after passage through an adherence column produced nearly twice as many clusters on macrophage monolayers as did the unpassed LNC from the same immune guinea pig. The most straightforward interpretation of this finding is that the lymphocytes involved in the formation of clusters are mainly, if not exclusively, T lymphocytes. The characterization of this subpopulation is the subject of further studies.

What is the role of macrophage-lymphocyte clusters in the immune response? As stressed above, functional studies have shown that macrophages and lymphocytes cooperate in the immune response (9, 11-18) and some of the evidence indicates that the cooperation requires physical contact between the two cells. The clusters described here may provide a microenvironment necessary for macrophage-lymphocyte cooperation mediated by surface contact and/or soluble factors.

Summary

We have studied the physical interaction between macrophages and lymphocytes during the immune response to purified protein derivative of tuberculin (PPD) in

vitro. Mixtures of peritoneal macrophages and lymph node lymphocytes from guinea pigs immunized with tubercle bacilli formed cell clusters during 20 h of culture with PPD. The number of clusters produced was correlated to the number of immune lymphocytes in the cultures. Peritoneal macrophages which had been pulsed with PPD and untreated lymph node lymphocytes produced cell clusters in the absence of free PPD in numbers equivalent to those produced by the same cells in the presence of free PPD. In cultures containing a mixture of PPD-pulsed macrophages, not-pulsed macrophages, and immune lymphocytes with no free PPD, cell clusters developed mainly between the antigen-pulsed macrophages and lymphocytes. Cluster formation was antigen-specific with the specificity residing in the lymphocytes, mainly or exclusively in the T lymphocytes. These data indicate that in the process of cell cluster formation macrophages serve as antigen-binding (or -processing) cells, while a subpopulation of lymphocytes interact physically and specifically with the macrophages.

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