MACROPHAGE-LYMPHOCYTE INTERACTION

I. Characteristics of the Antigen-Independent-Binding of Guinea Pig Thymocytes and Lymphocytes to Syngeneic Macrophages

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Physical approximation between lymphocytes and macrophages has been observed during cultivation of these cells (1–14), as well as when fixed preparations of lymphoid organs are examined (15–18). Although the exact nature of these cellular associations remains obscure, their occurrence both in vitro and in vivo, often in relationship to defined functional events, suggests physiologic significance.

In the intact animal, clustering of lymphocytes and blast cells about macrophages has been observed in lymph nodes responding to immunization procedures (16–18). Likewise, the in vitro induction of primary antibody responses (7, 19), as well as antigen-mediated in vitro proliferation of immune lymphocytes (4–6, 8, 12), has been observed to involve direct physical contact between lymphocytes and antigen-containing macrophages. Anatomic approximation, thus, may facilitate functional cooperation between macrophages and lymphocytes during the expression of an immune response.

Lymphocytes have also been found closely associated with macrophages in vivo in the absence of specific immunization (15, 17, 18) and in vitro when lymphoid cells from mouse (7, 13), rabbit (1, 12), guinea pig (9–11, footnote 1), or human (2, 3) are cultured without relevant antigen. This suggests that these cellular interactions may subserve a variety of biological functions not involving antigens such as the maintenance of lymphocyte viability (20), or the promotion of the functional maturation and differentiation of thymocytes (21). While the presence of antigen has been noted to affect the degree of macrophage-lymphocyte interaction (4, 6, 7, 11, 12, 17, 18), the occurrence of similar cellular associations in the absence of specific antigen suggests that the initiation of these contacts may be antigen-independent.

This report describes studies designed to elucidate the nature of the physical interaction between macrophages and lymphocytes. An in vitro method was developed for examining the binding of guinea pig nonglass-adherent lymphoid cells to syngeneic macrophage monolayers. This cellular association was characterized through the use of a variety of experimental conditions, inhibitors, and enzymes, and examined ultrastructurally. The binding of thymocytes and lymphocytes to macrophages required active macrophage metabolism but not anti-

[1] Lopez, L. R., K. S. Johansen, J. Radovich, and D. W. Talmage. 1973. The interaction of thymus derived cells with macrophages and erythrocytes. Manuscript in preparation.

gen. The requisite conditions for this interaction as well as the specificity of the participant cell types suggest the existence of a unique cellular recognition mechanism for the preferential association of macrophages and lymphocytes.

Materials and Methods

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

Media.—All washing procedures except where indicated were performed in Eagle's minimum essential medium (MEM)[2] for suspension cultures (Microbiological Associates, Bethesda, Md.) supplemented with fresh L-glutamine (0.3 mg/ml), glucose (5 mg/ml), sodium pyruvate (10 mM), nonessential amino acids 100 × (0.01 ml/ml; Grand Island Biological Co., Grand Island, N. Y.), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cultures were performed in supplemented MEM (SMEM) plus 10% heat-inactivated syngeneic normal guinea pig serum (GPS).

Cell Preparation.—All cells were prepared from unimmunized guinea pigs except where noted.

Glass-adherent cells: Peritoneal exudate cells (PEC) were harvested 3 days after the intraperitoneal injection of 20 ml sterile Marcol 52 (Humble Oil and Refining Co., Houston, Tex.) by lavage of the peritoneal cavity with 200 ml SMEM. Peritoneal macrophages (nonoil-induced) were similarly obtained by lavaging the peritoneal cavities of normal untreated guinea pigs. Spleen cell suspensions were prepared by teasing apart spleens and pressing them through a no. 60 mesh wire screen (W. S. Tyler Co., Mentor, Ohio). Alveolar macrophages were obtained by a modification of a previously described technique (22). The trachea was clamped to prevent aspiration and entered with a 19 gauge needle through which the tracheobronchial tree was lavaged *in situ* with SMEM. Polymorphonuclear leukocytes (PMN) were harvested by lavaging the peritoneal cavities 18 h after the intraperitoneal instillation of 15 ml sterile sodium caseinate (12% wt/vol, Difco Laboratories, Detroit, Mich.) (23). Each cell preparation was washed, exposed to isotonic ammonium chloride to lyse excess erythrocytes (24), washed three times with SMEM, counted, and resuspended in SMEM with 10% GPS for culture. Fibroblast monolayers were established from minced, trypsinized kidney tissue obtained from 3-wk old animals and used after 5–7 days of culture.

Nonglass-adherent cells: Thymocytes were obtained by teasing apart thymuses and pressing them through no. 60 mesh screens to obtain single cell suspensions. Lymph node lymphocyte (LNL) suspensions were prepared as previously described (25) by teasing and screening lymph nodes obtained from guinea pigs immunized 2–4 wk earlier with complete Freund's adjuvant (containing 2 mg/ml killed *M. tuberculosis* H37 Ra; Difco Laboratories). After exposure to ammonium chloride to remove erythrocytes, each population of cells was passed over glass-bead and nylon-wool columns (26) to deplete adherent cells. The resultant cells were more than 95% viable by Trypan blue dye exclusion and contained less than one macrophage per 100 cells by established criteria (26). L_2C leukemia cells, a gift of Dr. E. Shevach, were obtained by cardiac puncture from strain 2 guinea pigs previously inoculated with L_2C cells intradermally (27). Erythrocytes were obtained from guinea pigs by cardiac puncture, separated from the buffy coat, and washed in EDTA and SMEM as described (28). Each cell population was then washed three times in SMEM, counted, and resuspended in SMEM with 10% GPS (except where noted) for experimental use.

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[2] *Abbreviations used in this paper:* GPS, heat-inactivated syngeneic normal guinea pig serum; HBSS, Hanks' balanced salt solution; LNL, column-purified lymph node lymphocytes; Mϕ, macrophage; MEM, Eagle's minimum essential medium; PEC, peritoneal exudate cell; PMN, polymorphonuclear leukocyte; SMEM, supplemented Eagle's minimum essential medium for suspension culture.

Cell Counts.—All cell counts were performed with a Coulter Counter, Model FN (Coulter Electronics, Inc., Hialeah, Fla.).

Reagents.—The following reagents were used: 1X crystallized trypsin (Sigma Chemical Co., St. Louis, Mo.), *Vibrio cholerae* neuraminidase (General Biochemicals Div., Chagrin Fall, Ohio), iodoacetic acid, sodium salt (J. T. Baker Chemical Co., Phillipsburg, N. J.), sodium azide (Sigma Chemical Co.), 2-deoxy-D-glucose (Sigma Chemical Co.), mitomycin C (Nutritional Biochemicals Corp., Cleveland, Ohio), actinomycin D (Calbiochem, San Diego, Cal.), cyclohexamide (Calbiochem), puromycin (Calbiochem), disodium ethylenediaminetetraacetate (Fisher Scientific Co., Pittsburgh, Pa.), and Trypan blue stain in normal saline (Grand Island Biological Co.).

Culture Chambers.—In vitro cultures were performed using microscope slide/tissue culture chambers (Lab-Tek Products, Division of Miles Laboratories, Kankakee, Ill.). These chambers consist of a glass microscope slide base with sides and lid formed by a removable plastic superstructure. At the conclusion of an experiment, the plastic superstructure was removed and the resultant microscope slide with its adherent cells processed for light or electron microscopy.

Culture Technique.—Macrophage monolayers were prepared by culturing 1×10^6 cells in each microscope slide/culture chamber in 1 ml of SMEM with 10% GPS at 37°C in an atmosphere of 5% CO_2 and 95% air. After 3 h in culture, nonglass-adherent cells were removed by two washes with SMEM. 2 ml of SMEM with 10% GPS were added to each of the chambers and the glass-adherent cells incubated overnight at 37°C in a 5% CO_2, 95% air environment. The following day, any additional nonadherent cells were removed with two washes of SMEM and the monolayers immediately used for experiments. PMN were likewise cultured at a concentration of 1×10^6 per chamber in 1 ml of SMEM with 10% GPS. After 90 min in culture at 37°C any nonadherent cells were removed by two washes with SMEM. The glass-adherent PMN were then immediately used.

Macrophage-Lymphocyte Association.—Single cell suspensions of thymocytes, lymph node lymphocytes, L_2C leukemia cells, or erythrocytes were added to the monolayers in the slide/chambers in 2 ml of SMEM + 10% GPS. The chambers were incubated at 37°C in a 5% CO_2, 95% air atmosphere on a slowly rocking platform (Bellco Glass, Inc. Vineland, N. J.) for various periods of time as indicated in the protocol. At the conclusion of an experiment, unbound cells were removed by decanting the supernatants and dipping and swirling the slides in three changes of SMEM with 1% GPS. The macrophage monolayers with cells adherent to them were fixed in 1% glutaraldehyde in modified Tyrodes buffer for 30 min and stained with buffered Giemsa in acetone. After the plastic superstructure of the chambers was removed, cover slips were applied to the slides and observations made using a Zeiss photomicroscope II (Carl Zeiss, Inc., New York).

Enzyme Treatment.—Macrophage monolayers which had previously been cultured for 24 h in vitro were washed three times with Hanks' balanced salt solution (HBSS) and exposed to trypsin (0.4 mg/ml) in HBSS for 20 min at room temperature or to neuraminidase (50 U/ml) for 60 min at 37°C. They were then washed with three changes of cold SMEM with 10% serum and immediately used. After either treatment viability remained greater than 95% by Trypan blue exclusion.

Suspensions of 20×10^6 column-purified thymocytes were washed three times in HBSS and exposed to either trypsin (0.4 mg/ml) for 20 min at room temperature or neuraminidase (50 U/ml) for 60 min at 37°C in suspension. Cells were then washed three times with cold SMEM with 10% GPS, suspended in SMEM with 10% GPS and immediately used. Viability by Trypan blue exclusion remained greater than 95%.

Heat-Killing.—Established macrophage monolayers were heated at 56°C for 60 min, washed two times and immediately used. Column-purified thymocytes were exposed to 56°C for 60 min in suspension in HBSS, washed twice, suspended in SMEM with or without 10% GPS and immediately used. Cells treated in this manner were uniformly nonviable by Trypan blue exclusion.

Electron Microscopy.—Monolayers were fixed in 1% glutaraldehyde with Tyrodes buffer for 30 min. After postfixation with 1% osmium tetroxide, adherent cells were dehydrated directly on the glass slides and embedded in Maraglas (Polyscience Corp., Evanston, Ill.) using inverted Beem capsules. After polymerization for 48 h at 65°C the Maraglas blocks were separated from the glass slides by immediate immersion in liquid nitrogen. Thin sections were prepared, stained with lead citrate and uranyl acetate, and examined with a Philips 300 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.).

RESULTS

Macrophage Monolayers.—Monolayers, cultivated on glass for 24 h in vitro provided a population of uniformly viable cells (>95% by Trypan blue exclusion) whose characterixtics could be defined both morphologically and physiologically by glass adherence and phagocytosis. The number of macrophages comprising a 24-h old monolayer could be directly counted microscopically and was found to reflect the number of peritoneal exudate cells initially cultured such that 1×10^6 PEC yielded 1×10^5 (range = $0.7 - 1.3 \times 10^5$) adherent cells per slide/chamber. This density of macrophages was most convenient for accurate counting of lymphocytes clustered about individual macrophages. After 1 h of culture, certain cell populations, especially spleen cells, were found to have formed spontaneous clusters of lymphoid cells about larger glass adherent cells. When, however, the monolayers were examined microscopically after 24 h in culture, less than one lymphocyte was found per 100 macrophages (Fig. 1 *A*).

Binding of Thymocytes To Macrophage Monolayers; Quantitative Considerations.—Varying numbers of column-purified thymocytes (0.1×10^6 to 10×10^6) were added to macrophage monolayers in 2 cc of SMEM with 10% GPS and allowed to incubate at 37°C for 1 h with gentle rocking. After unbound cells were washed away and slides prepared, clustering of thymocytes about macrophages was routinely observed (Fig. 1 *B* and 1 *C*). The majority of thymocytes was found aligned along the perimeter of macrophages. Less than 5% of the thymocytes associated with monolayers had been internalized into macrophages. There was no relationship between the morphological characteristics of a macrophage and its likelihood of having one or more thymocytes associated with it, although larger macrophages tended to have more thymocytes associated with them.

Macrophage-thymocyte association was quantified by counting the number of thymocytes in physical contiguity with each of 200 randomly chosen macrophages. The data obtained were expressed as either (*a*) the percent of macrophages found associated with one or more thymocytes, or (*b*) the total number of thymocytes bound to 100 macrophages. While concomitant effects were usually observed in both of these parameters, the latter was found to be more sensitive to experimental manipulation. Thus, the data from many experiments will be presented only in terms of the number of thymocytes bound per 100 macrophages. Fig. 2 describes the relationship between the number of added

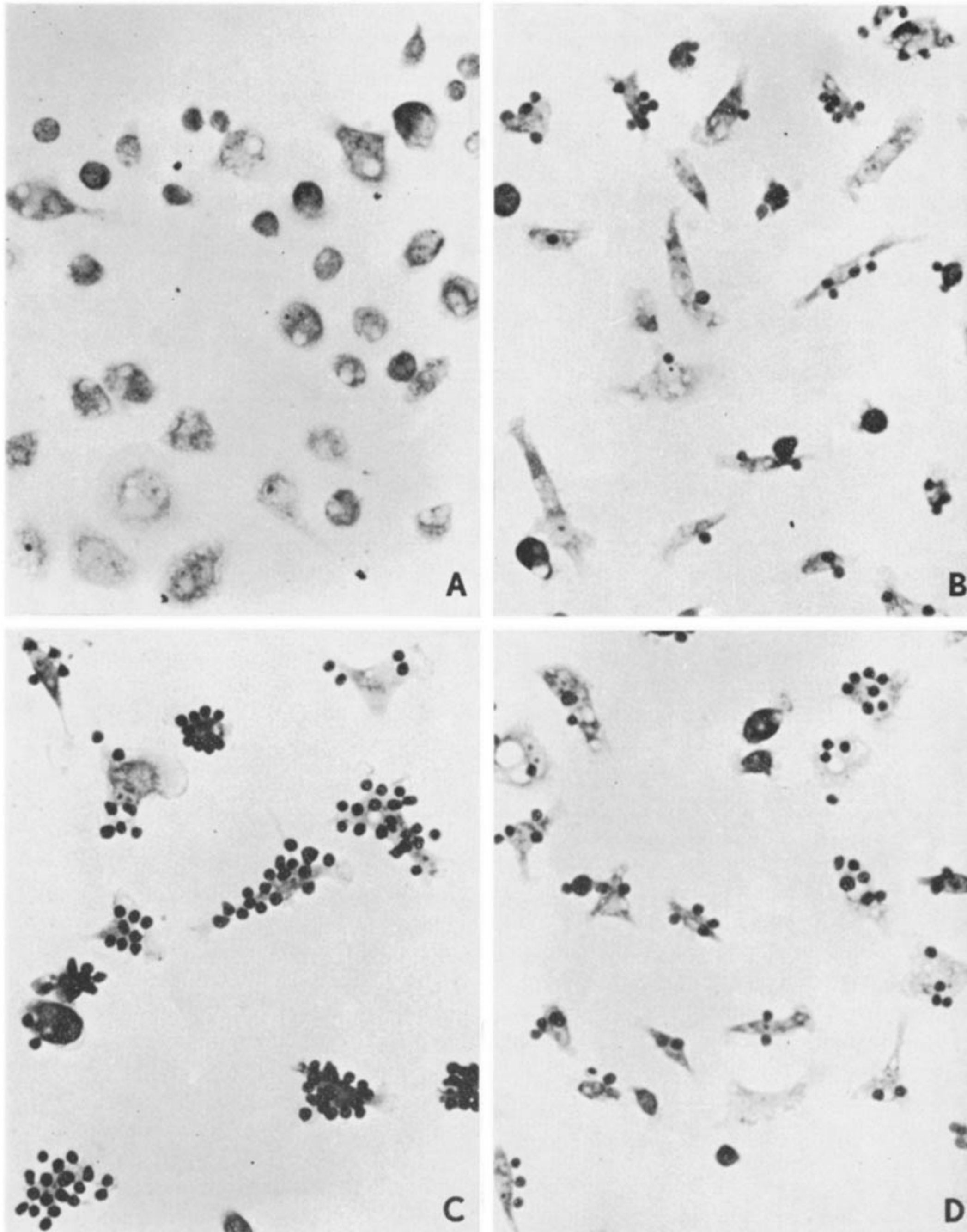
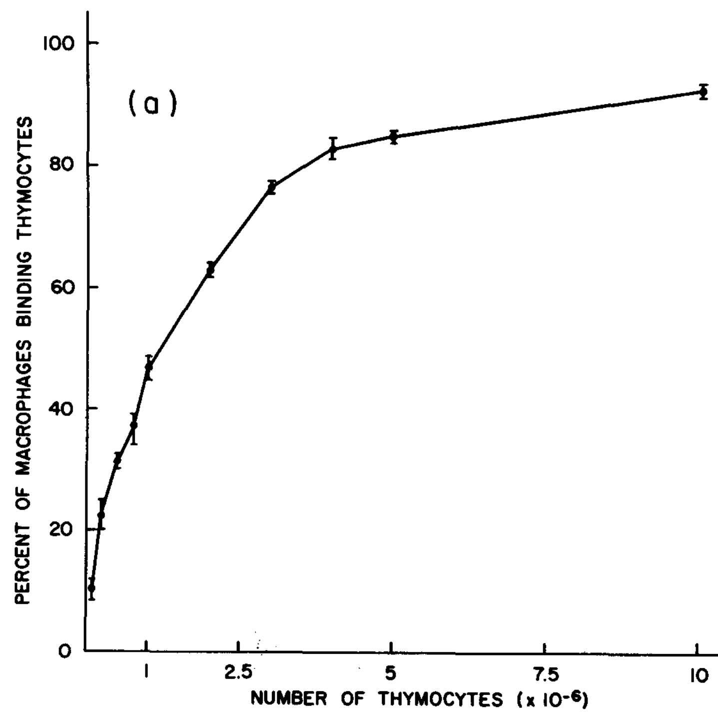


FIG. 1. The binding of column-purified guinea pig thymocytes to syngeneic macrophage monolayers in microscope slide/culture chambers. (*A*) Macrophage monolayers obtained by culturing peritoneal exudate cells for 24 h in SMEM plus 10% GPS. Before the addition of thymocytes, less than one lymphocyte per 100 macrophages was observed. (*B*) Clustering of thymocytes about macrophages observed when 2×10^6 thymocytes were added to monolayers in 2 ml SMEM plus 10% GPS and incubated for 60 min at 37°C. The vast majority of bound thymocytes were found aligned along the free surfaces of macrophages. Very few thymocytes have been internalized into macrophages. (*C*) Thymocyte-binding observed when 10×10^6 thymocytes were added to syngeneic monolayers and incubated in standard fashion. (*D*) The binding of heat-killed thymocytes (2×10^6) to viable macrophage monolayers. Few of the bound, heat-killed thymocytes have been internalized. Magnification $\times$ 800.



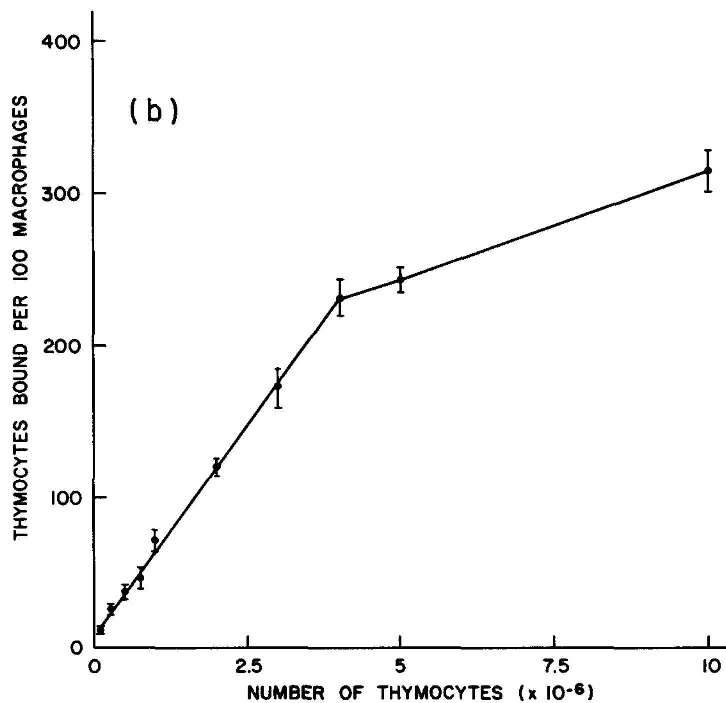


FIG. 2. The binding of guinea pig thymocytes to glass-adherent macrophages as a function of the number of thymocytes presented. For each experiment the number of thymocytes found in physical contiguity with each of 200 macrophages was counted and the data expressed as (*a*) the percent of macrophages found associated with one or more thymocytes and (*b*) the total number of thymocytes bound to 100 macrophages. Each point represents the mean of four or more replicate experiments with vertical bars indicating the standard errors.

thymocytes and each of the above noted parameters. The maximum number of cells added per chamber, 10×10^6, resulted in one or more thymocytes being associated with more than 90% of the macrophages and a total of 316 ± 13 thymocytes bound per 100 macrophages. When 2×10^6 thymocytes were added, 63% of macrophages were found associated with thymocytes with 120 ± 5 bound per 100 macrophages. Because 2×10^6 added thymocytes yielded values for each parameter which were well within the most linear part of each of these curves, this number of thymocytes was used for most comparative experiments.

The total number of thymocytes binding to a monolayer could be calculated from the number of thymocytes bound per 100 macrophages and the direct count of the number of macrophages comprising the monolayer. Thus, when the number of thymocytes presented to standard monolayers was increased (0.5×10^6 through 5.0×10^6) the total number of thymocytes bound to the monolayers increased. Despite this, the percentage of added thymocytes bound to the monolayers remained constant (5.9 ± 0.4%). This observation suggested that only a small subset of thymocytes was capable of physically interacting with macrophages. Alternatively, the physical characteristics of the incubation system could have been important in dictating the degree of observable interaction. To explore this question, the geometry of the system was altered by varying the number of macrophages composing the monolayers. If only 6% of a thymocyte population were capable of binding to macrophages, then changing the density of cells in the monolayer should not result in an increased percentage-binding of added thymocytes. On the other hand, if a greater percentage of thymocytes could bind, increasing the density of the monolayer should increase the likelihood of cellular interaction and thus lead to an increase in the percentage of added thymocytes bound. When 2×10^6 thymocytes were presented to monolayers of varying macrophage densities under standard conditions (Fig. 3), the percentage of added thymocytes bound to the monolayers increased as the number of macrophages composing the monolayer increased. Thus, it is more likely that the percentage-binding observed is reflective of the conditions of the incubation system rather than of the existence of a specialized subset of macrophage-associating thymocytes.

Binding of Thymocytes To Macrophage Monolayers—Effect of Variation in Experimental Conditions.—The standard experiments measured the physical association which occurred between 2×10^6 thymocytes and 24-h old macrophage monolayers after a 60 min incubation at 37°C with gentle rocking in the presence of SMEM with 10% GPS. The presence of serum was not found to play a significant role in thymocyte-macrophage interaction (Table I). The possibility that cytophilic immunoglobulin bound onto the macrophage was mediating thymocyte-binding was tested by culturing the macrophages overnight in SMEM with 10% immunoglobulin-free fetal calf serum. The monolayers were washed three times before use and after each wash allowed to incubate in the presence of SMEM with 10% immunoglobulin-free fetal calf

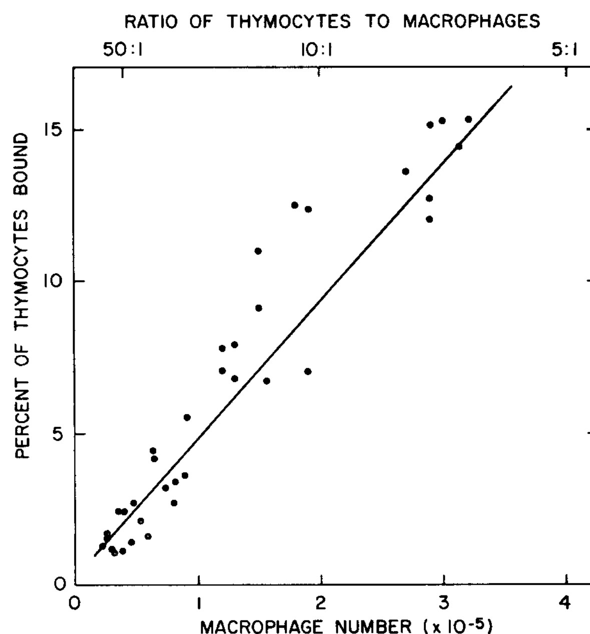


FIG. 3. The relationship between the number of macrophages composing a monolayer and the percentage of added thymocytes bound after a 1 h incubation. The number of macrophages comprising an adherent-cell monolayer was counted microscopically. When 2×10^6 syngeneic column-purified thymocytes are added to monolayers composed of 2.2×10^4 to 3.2×10^5 macrophages and incubated for 60 min at 37°C with gentle rocking, the percent of added thymocytes bound by a monolayer is = (mean number of thymocytes per macrophage) (macrophages per monolayer)/(total thymocytes added) $\times$ 100.

serum for 30 min at 37°C. After these washes, significant amounts of immunoglobulin could not be detected on the macrophage surface utilizing rabbit antiguinea pig immunoglobulin tagged with horseradish peroxidase as a marker.[3] Monolayers thus depleted of cytophilic immunoglobulin were as capable of binding thymocytes as control macrophages. Likewise, the presence of SMEM itself was found not to be essential in that no decrease in cellular association occurred when the reaction was run in HBSS in the absence of serum. Rocking increased macrophage-thymocyte association by a small, but significant, degree when 2×10^6 thymocytes were presented to macrophage monolayers. When, however, the number of added thymocytes was increased to 10×10^6, the effect of rocking became insignificant.

In vitro phagocytosis by macrophages was found to have little effect on thymocyte-binding (Table I). When macrophage monolayers were exposed to 0.1% polystyrene latex particles (1.1 μm diameter, 1.05 g/ml, Dow Chemical

[3] Lipsky, P. E., and A. S. Rosenthal. 1973. Manuscript in preparation.

TABLE I

The Effect of Variations in Experimental Conditions on the Degree of Macrophage-Thymocyte Association

Variable	Percent of macrophages-binding thymocytes	Thymocytes bound per 100 macrophages
	% of control*	
Serum‡		
(*a*) No serum	101.1 ± 1.2	97.8 ± 4.8
(*b*) 50% guinea pig serum	101.5 ± 1.6	100.9 ± 1.9
(*c*) 10% fetal calf serum	94.7 ± 1.3	95.5 ± 3.5
Rocking		
Stationary incubation	91.3 ± 1.4	77.8 ± 4.1
Phagocytosis by macrophages§		
Before	98.3 ± 2.2	99.1 ± 2.6
Concurrent	95.8 ± 3.5	93.0 ± 7.7
In vitro cultivation‖ of macrophages		
1 h	61.5 ± 2.7	34.5 ± 2.3
48 h	107.2 ± 1.8	132.3 ± 13.6
96 h	113.6 ± 1.2	157.0 ± 5.3

* Data expressed as a percentage of the macrophage-thymocyte association observed when 2×10^6 column-purified (viability >95%) thymocytes were added to syngeneic macrophage monolayers (derived by culturing peritoneal exduate cells in vitro for 24 h) in 2 ml SMEM with 10% GPS and incubated for 60 min at 37°C with gentle rocking. Each term represents the mean ± standard error of three or more replicate experiments.

‡ Standard monolayers presented with 2×10^6 thymocytes in (*a*) 2 ml SMEM alone, (*b*) 2 ml SMEM with 50% GPS, (*c*) 2 ml SMEM with 10% immunoglobulin-free fetal calf serum after manipulations to remove cytophilic immunoglobulin from the surface of macrophages (see text).

§ Monolayers challenged with 0.1% polystyrene latex particles before or concomitant with standard thymocyte presentation.

‖ Duration of in vitro cultivation of macrophages before standard thymocyte presentation.

U.S.A. Membrane Systems Div., Midland, Mich.) for 30 min at 37°C, 78 ± 3% of the macrophages were found to have ingested three or more beads. No significant effect on macrophage-thymocyte association was observed when macrophages had ingested beads before being exposed to thymocytes or had beads and thymocytes presented simultaneously. Macrophages which had not ingested polystyrene latex beads were as likely to bind thymocytes as those which had ingested beads.

The length of time that the macrophages were cultivated in vitro before use markedly affected the observed degree of macrophage lymphocyte interaction (Table I). Shortly after glass-adherence, macrophages demonstrated less thymocyte-binding than cells which had been cultured on glass for 24 h. As time in culture was prolonged to 48 and 96 h, increases in binding ability were observed above that seen with 24-h old cells.

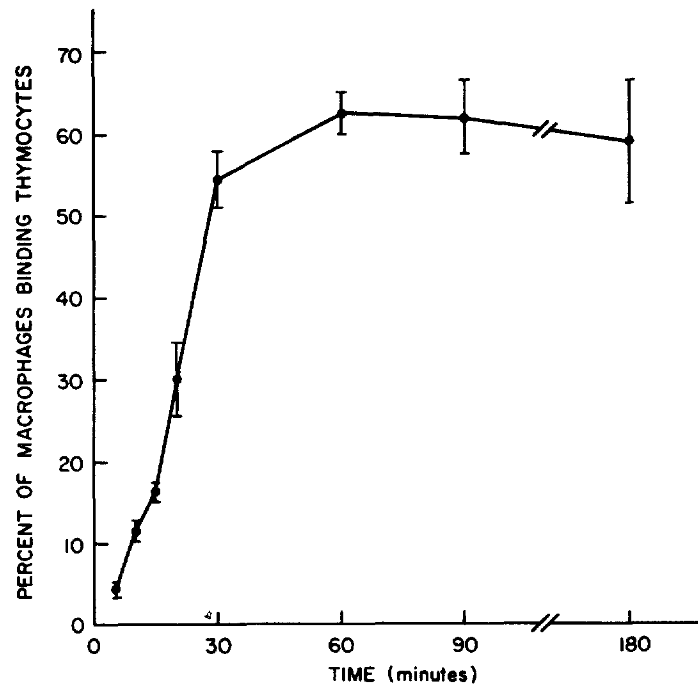
Binding of Thymocytes to Macrophage Monolayers—Kinetic Considerations.—

The interaction of thymocytes and macrophages was a time-dependent phenomenon. Fig. 4 shows the binding of 2×10^6 thymocytes to macrophage monolayers as a function of the time during which the two cell types were incubated together. Maximal binding occurred within 60 min in culture and was maintained for up to 180 min. Within 30 min the percent of macrophages binding one or more thymocytes was 87% of maximal while the number of thymocytes bound per 100 macrophages was 82% maximal. Because of the kinetics observed, 60 min was chosen as the standard incubation time for all further experiments.

Binding of Thymocytes and Lymphocytes to Glass-Adherent Cell Populations—Specificity of the Participating Cells.—The binding of thymocytes to a variety of glass-adherent cell populations was investigated to delineate the cellular specificity of this reaction (Fig. 5). Nonoil-induced peritoneal macrophages bound significantly fewer ($P < 0.01$) thymocytes per 100 glass-adherent cells than PEC when 2×10^6 thymocytes were presented. However, at higher numbers of added thymocytes there was no significant difference between the number of thymocytes bound per 100 nonoil-induced peritoneal macrophages or 100 PEC. The binding of thymocytes to splenic macrophages was not significantly different from that to PEC. Alveolar macrophages bound significantly fewer thymocytes than PEC at all numbers of added thymocytes. Polymorphonuclear leukocytes were found associated with only about 15% as many thymocytes as PEC at all numbers of added thymocytes. Furthermore, PMN were significantly less capable ($P < 0.001$) of binding thymocytes than either freshly harvested PEC or PEC which had been cultured for 1–2 h. Fibroblasts bound only about 15% as many thymocytes as PEC at all numbers of added thymocytes. All four of the macrophage populations were capable of binding significantly more ($P < 0.001$) thymocytes per 100 adherent cells than any of the nonmacrophage populations of cells.

The ability of macrophage monolayers to bind a variety of non-glass-adherent cell types was investigated (Table II). LNL were bound to macrophage monolayers much in the same fashion as thymocytes but in reduced numbers. The time requirement for LNL-binding to macrophage monolayers was quite similar to that observed for thymocytes. Maximal binding was achieved by 60 min and lasted for up to 3 h. Binding of LNL to macrophage monolayers was similar in SMEM with 10% GPS, SMEM with 10% immunoglobulin-free fetal calf serum, or SMEM alone. The association of L_2C leukemia cells with macrophage monolayers was significantly less than that seen with LNL or thymocytes in the presence or absence of guinea pig serum. No significant binding of guinea pig erythrocytes by macrophage monolayers was observed.

Influence of Temperature on Macrophage-Thymocyte Association.—In these experiments each macrophage monolayer was equilibrated at experimental temperatures for 15 min before the addition of 3×10^6 thymocytes. The interaction of macrophages and thymocytes was temperature-dependent with bind-



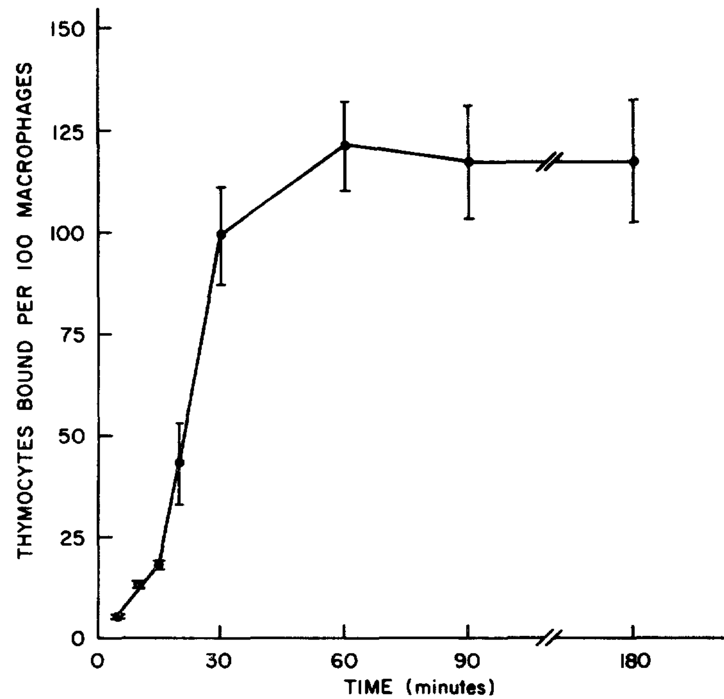


FIG. 4. The kinetics of the physical interaction between glass-adherent peritoneal exudate macrophages and syngeneic thymocytes. Column-purified thymocytes (2×10^6) were added to standard macrophage monolayers in 2 ml SMEM with 10% GPS and incubated at 37°C with gentle rocking for varying periods of time. Each point represents the mean of four or more replicate experiments with standard errors indicated by the vertical bars.

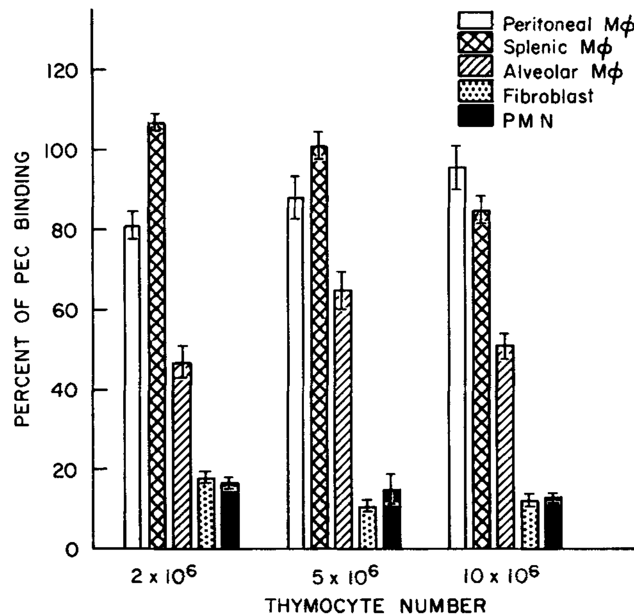


FIG. 5. The ability of a variety of populations of glass-adherent cells to bind thymocytes. The height of each bar indicates the mean of four or more replicate experiments and the bracket the standard error. The data represent the total number of thymocytes found physically associated with 100 glass-adherent cells expressed as a percent of the binding observed when equal numbers of thymocytes were presented to macrophage monolayers derived from PEC.

ing occurring most efficiently at 37°C (Fig. 6). The effect of temperature could not be ascribed to decreased viability of the macrophages maintained at sub-optimal temperatures as judged by Trypan blue exclusion. There was no statistically significant difference in the maximum association observed at 37°C or 20°C, although the time required to achieve that maximum was markedly temperature sensitive, increasing from 60 min to 120 min as the temperature was decreased from 37°C to 20°C. The maximum thymocyte-macrophage association observed at 4°C, even after a 3 h incubation, was significantly less ($P < 0.01$) than that observed with either 37°C or 20°C incubations.

Ability of Heat-Killed Cells to Participate in Macrophage-Thymocyte Association.—While heat-killed macrophage monolayers were incapable of participating in significant macrophage thymocyte interaction (Table III), heat-killed thymocytes did bind to viable macrophages (Fig. 1 *D*). Similar degrees of association were seen between heat-killed thymocytes and macrophage monolayers in the presence or absence of heat-inactivated guinea pig serum. It was noted that with incubation of up to 3 h, less than 13% of the heat-killed thymocytes bound by viable macrophages had been internalized.

TABLE II

The Ability of Various Nonglass-Adherent Cell Types to Bind to Macrophage Monolayers

Cell no.	Cell type*	Percent of macrophages-binding cells	Cells bound per 100 macrophages
2×10^6	Thymocyte	62.9 ± 1.8	120 ± 5
	LNL	36.8 ± 3.8 (58.5%)‡	49 ± 7 (40.8%)
	L_2C	6.8 ± 1.1 (10.8%)	8 ± 1 (6.7%)
	RBC	0.8 ± 0.3 (1.3%)	1 ± 0 (0.8%)
5×10^6	Thymocyte	85.2 ± 1.0	244 ± 7
	LNL	53.1 ± 2.8 (62.3%)	95 ± 9 (38.9%)
	L_2C	13.9 ± 1.3 (16.3%)	18 ± 2 (7.4%)
	RBC	3.3 ± 1.5 (3.9%)	3 ± 1 (1.2%)
10×10^6	Thymocyte	91.9 ± 1.0	316 ± 13
	LNL	58.9 ± 5.1 (64.1%)	118 ± 20 (37.3%)
	L_2C	20.0 ± 1.5 (21.8%)	29 ± 5 (8.1%)
	RBC	2.8 ± 1.5 (3.0%)	3 ± 1 (0.9%)

* Preparation of cells is described in Materials and Methods. Indicated number of each cell type was added to individual syngeneic macrophage monolayers in 2 ml SMEM with 10% GPS and incubated for 60 min at 37°C with gentle rocking. Each term represents the mean ± standard error of four or more replicate experiments.

‡ Expression in parentheses indicates binding of each cell type expressed as a percentage of the binding observed when an equal number of thymocytes was added to macrophage monolayers under standard conditions.

TABLE III

The Ability of Heat-Killed Cells to Participate in Macrophage-Thymocyte Association

Heat-killed cell	Percent of macrophages-binding thymocytes	Thymocytes per 100 macrophages
	% of control*	
Macrophage‡	6.5 ± 1.1	2.9 ± 0.5
Thymocyte§	96.6 ± 3.7	122.4 ± 9.4

* Data expressed as a percentage of the macrophage-thymocyte association observed when 2×10^6 column-purified (viability >95%) thymocytes were incubated with syngeneic macrophage monolayers (viability >95%) under standard conditions. Each term represents the mean ± standard error of eight or more replicate experiments.

‡ Established macrophage monolayers were heated at 56°C for 60 min before the presentation of 2×10^6 thymocytes (viability >95%) in standard fashion.

§ Column-purified thymocytes were heated at 56°C for 60 min, washed, counted, and presented to untreated macrophage monolayers and incubated for 60 min under standard conditions.

Effect of Various Inhibitors on Macrophage-Thymocyte Interaction.—2-Deoxyglucose, a reversible inhibitor of glycolysis, thought to affect the efficiency of glucose utilization (24), caused a small, but significant ($P < 0.02$) decrease in the number of thymocytes bound per 100 macrophages when 2×10^6 thymocytes were added to pretreated macrophages in the presence of the inhibitor

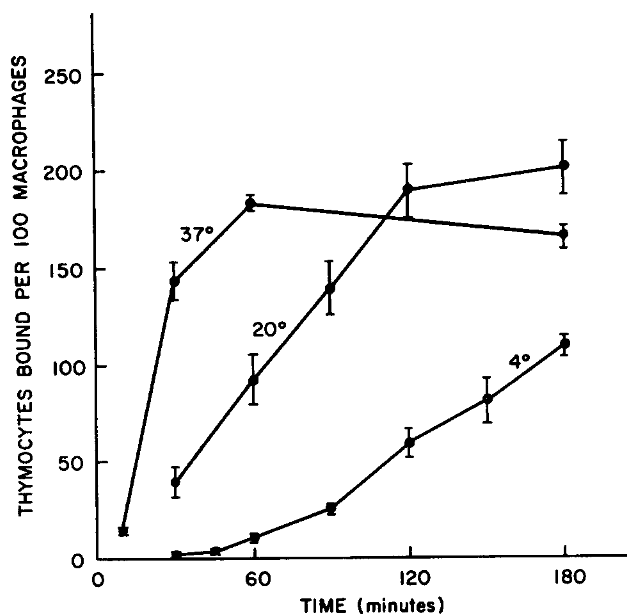


FIG. 6. The effect of temperature on the physical association between thymocytes and macrophages. Column-purified thymocytes (3×10^6) were incubated with syngeneic macrophage monolayers for 10–180 min with gentle rocking at 4°C, 20°C, or 37°C. Each point indicates the number of thymocytes bound per 100 macrophages and represents the mean of four replicate experiments with the vertical bars showing the standard errors. The maximal binding noted at 20°C was not significantly different from that observed at 37°C.

(Table IV). No change in viability of treated macrophages was observed. Macrophages, which had been pretreated with 2-deoxyglucose and were then presented with thymocytes in the absence of the inhibitor, exhibited no significant decrease in binding as compared to control macrophages. Sodium azide, a reversible inhibitor of oxidative phosphorylation, also significantly decreased binding, without affecting viability, when thymocytes (2×10^6) were added to pretreated macrophages in the presence of the inhibitor. The combination of sodium azide + 2-deoxyglucose, when present continuously, resulted in nearly complete inhibition of thymocyte macrophage association. Sodium iodoacetate (3×10^{-3} M), an irreversible inhibitor of glycolysis, markedly decreased thymocyte binding when thymocytes (2×10^6) were added to pretreated macrophages in the presence or absence of this inhibitor (Table IV). Viability of the macrophages as gauged by Trypan blue exclusion was not affected. Thymocytes, on the other hand, which had been pretreated for 1 h with 3 mM sodium iodoacetate (posttreatment viability >95%) exhibited a normal degree of binding to untreated monolayers.

Inhibitors of protein synthesis (puromycin, cyclohexamide), DNA (mitomycin C), or RNA (actinomycin D) metabolism had no significant effect on

TABLE IV

The Effect of Metabolic Inhibitors on Macrophage-Thymocyte Association

Inhibitor conc.	Thymocytes-bound per 100 macrophages % of control*
2-Deoxyglucose‡	
(20 mM)	76.4 ± 4.1
Sodium azide§	
(20 mM)	25.6 ± 4.3
Sodium azide + 2-deoxyglucose‖	
(20 mM + 20 mM)	4.1 ± 0.4
Sodium iodoacetate	
(3 mM)	
Continuous¶	10.5 ± 0.9
Macrophage**	19.8 ± 4.0
Thymocyte‡‡	105.8 ± 6.2

* Data expressed as a percentage of the macrophage-thymocyte association observed when 2×10^6 column-purified thymocytes were incubated for 60 min at 37°C with macrophage monolayers in the absence of inhibitor. Each term represents the mean ± standard error of four replicate experiments.

‡ Monolayers pretrested with 2-deoxyglucose (20 mM) in HBSS without glucose for 30 min at 37°C. 2×10^6 thymocytes then presented in 2 ml HBSS without glucose + 2-deoxyglucose (20 mM) and incubated in standard fashion. Controls were run in HBSS without glucose. Pretreated macrophages presented with thymocytes in HBSS without inhibitor yield 89.4 ± 2.9% of control binding.

§ Monolayers pretreated with sodium azide (20 mM) in HBSS for 30 min at 37°C. 2×10^6 thymocytes presented in 2 ml HBSS + azide (20 mM) and incubated in standard fashion. Control run in HBSS. Pretreated macrophages presented with thymocytes in HBSS without inhibitor yield 100.9 ± 6.3% of control binding.

‖ Monolayers pretreated with 2-deoxyglucose (20 mM) + azide (20 mM) in HBSS without glucose for 30 min at 37°C. 2×10^6 thymocytes presented in 2 ml HBSS without glucose + both inhibitors and incubated in standard fashion. Pretreated macrophages presented with thymocytes in absence of inhibitor yields 73.5 ± 1.6% of control binding.

¶ Monolayers are pretreated with iodoacetate (3 mM) in SMEM for 30 min at 37°C. 2×10^6 column-purified thymocytes were then presented in 2 ml (SMEM with 10% GPS) plus iodoacetate (3 mM) and incubated in standard fashion.

** Monolayers were pretreated with iodoacetate (3 mM) in SMEM for 30 min at 37°C and washed times three. 2×10^6 thymocytes were then presented in 2 ml (SMEM with 10% GPS) containing no inhibitor and incubated in standard fashion.

‡‡ Column-purified thymocytes were pretreated with iodoacetate (3 mM) in SMEM in suspension for 30 min at 37°C, washed, and counted. (Viability after treatment >95%.) 2×10^6 treated thymocytes were presented to normal monolayers in 2 ml (SMEM with 10% GPS) containing no inhibitor and incubated in standard fashion.

macrophage-thymocyte interaction when macrophages, pretreated with inhibitor for 60 min, were presented with thymocytes in the presence of the inhibitor.

The Requirement for Divalent Cations in Macrophage-Thymocyte Interaction.— To assess the requirement for divalent cations, binding experiments were performed in SMEM with 10% GPS which contained 1×10^{-3} M disodium

EDTA. Higher concentrations of EDTA uniformly caused the macrophages to detach from the glass and thus could not be used. When 1 mM EDTA was present for 15 min before and during thymocyte presentation, the binding of thymocytes by macrophages was significantly ($P < 0.001$) reduced (Table V). Macrophage-thymocyte association in the presence of 1×10^{-3} M EDTA + 2×10^{-3} M Ca++ ($CaCl_2$) was not significantly different than control. Macrophage-binding of thymocytes in the presence of 1 mM EDTA + Mg++ ($MgSO_4$) at a concentration of 1 or 2 mM was significantly ($P < 0.02$) greater than in the situation when EDTA alone was present but significantly ($P < 0.001$) less than when 1 mM EDTA + 2 mM Ca++ were present (Table V).

Effect of Enzyme Treatment of the Participant Cells on Thymocyte-Macrophage Interaction.—Trypsin-treated thymocytes bind to normal macrophages in somewhat greater numbers than control thymocytes (Table VI). However, trypsin treatment of macrophages markedly decreased their ability to bind

TABLE V
The Role of Divalent Cations in Macrophage-Thymocyte Association

EDTA*	Cation‡	Conc.	Thymocytes bound per 100 macrophages
			% of control‖
1 mM	—	—	34.8 ± 1.5
1 mM	Mg++	1 mM	59.9 ± 8.8
1 mM	Mg++	2 mM	43.4 ± 4.1
1 mM	Ca++	1 mM	58.5 ± 4.2
1 mM	Ca++	2 mM	86.5 ± 4.4

* Macrophage monolayers were treated for 15 min at room temperature with 1 ml (SMEM + 10% GPS) with 1×10^{-3} M disodium EDTA. 2×10^{6} thymocytes were added to monolayers in 2 ml (SMEM with 10% GPS) + 1×10^{-3} M disodium EDTA + indicated cation at concentration noted. When EDTA was removed after pretreatment and thymocytes were added in 2 ml of SMEM with 10% GPS-binding was 85.2 ± 3.2 of control.

‡ Ca++ added as $CaCl_2$. Mg++ added as $MgSO_4$.

‖ Data expressed as a percentage of the standard macrophage-thymocyte association. Terms represent mean ± standard error of four or more replicate experiments.

TABLE VI
The Ability of Enzyme-Treated Cells to Participate in Macrophage-Thymocyte Association

Enzyme (conc.)	Cell treated	Thymocytes-bound per 100 macrophages
		% of Control*
Trypsin (0.4 mg/ml)	Macrophage	20.4 ± 4.2
	Thymocyte	125.5 ± 7.5
Neuraminidase (50 U/ml)	Macrophage	111.2 ± 5.0
	Thymocyte	205.9 ± 8.0

* Data expressed as a percentage of standard macrophage-thymocyte association. Each term represent the mean ± standard error of eight or more experiments.

thymocytes, whether the thymocytes were added in the presence or absence of serum. When trypsin-treated macrophages were cultured, their ability to bind thymocytes was slowly regained such that macrophages cultured for 24 h after trypsinization could bind 93.0 ± 3.1% as many thymocytes per 100 macrophages as control macrophages.

Macrophages, which had been treated with neuraminidase, showed a modestly increased ($P < 0.05$) ability to bind thymocytes (Table VI). Neuraminidase-treated thymocytes, on the other hand, were bound by macrophages in significantly greater ($P < 0.001$) numbers than control thymocytes. There was no increase in the endocytosis of neuraminidase-treated thymocytes as compared to untreated thymocytes in these experiments.

Ultrastructure of Macrophage-Thymocyte Association.—Electron microscopic observations of macrophage thymocyte interactions revealed that extremely close contact occurred between these cell-types, often with long expanses of closely approximated plasma membranes (Figs. 7 and 8). There was no obvious specialization of the submembrane architecture in the areas of the macrophage adjacent to thymocyte contact. Similarly, no particular region of the thymocyte was more frequently observed to be in contact with the macrophage. The vast majority of thymocytes were aligned along the surface of the macrophage in seemingly random fashion. Few, if any intact thymocytes were found to have been internalized into macrophages. Tangentially cut sections occasionally caused thymocytes to appear to be completely surrounded by macrophage cytoplasm, but serial sections revealed these cells to be external to the macrophage.

DISCUSSION

These studies were undertaken to characterize the physical interaction between lymphocytes and macrophage monolayers in vitro. The observations of Siegel (9, 10) that thymocyte "rosettes" developed about fresh guinea pig peritoneal mononuclear cells when the two cell types were mixed in suspension, our own observations of spontaneous clustering of lymphoid cells about larger glass-adherent cells in fresh spleen cell preparations as well as the numerous observations made on fixed sections of whole lymphoid organs (15, 17, 18) strongly suggest that the physical interaction between macrophages and lymphocytes studied in our system is representative of a function these cells possess in vivo and not one developed *de novo* during in vitro cultivation. The ability of the macrophage to bind thymocytes increased as the time in culture, before thymocyte presentation, was lengthened. This may reflect more uniform viability of the glass-adherent cells, maturation of a functional and/or metabolic potential or merely increased surface area of the cells. Mononuclear cells have also been observed to develop increased capacity for phagocytic uptake when maintained in prolonged culture (29). Likewise, macrophages, after extended in vitro cultivation, have been noted to be more capable

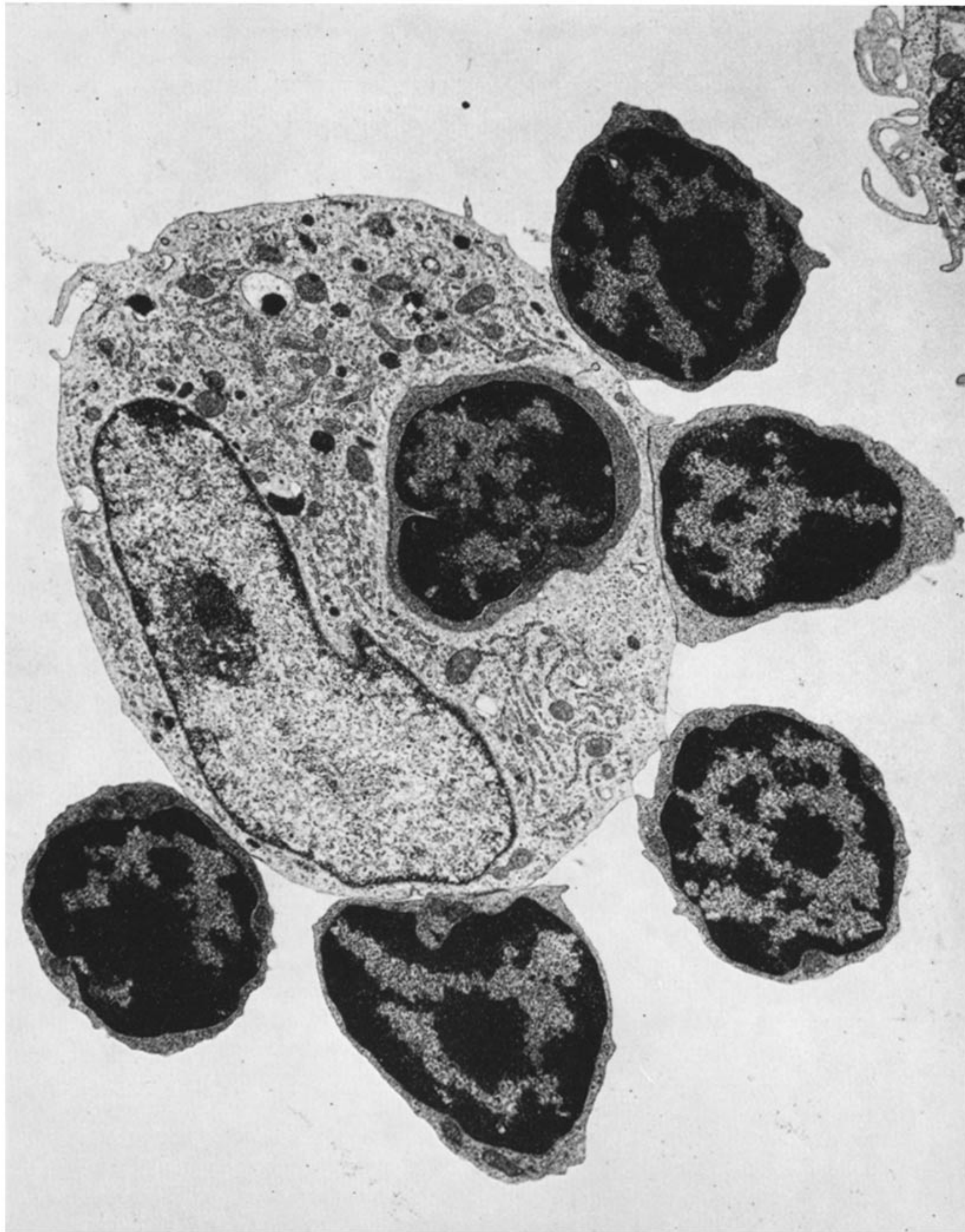


FIG. 7. The clustering of guinea pig thymocytes about syngeneic glass-adherent macrophages. Broad areas of approximation between these cells were observed with little obvious specialization of the architecture adjacent to areas of contact. These cells were oriented by virtue of having been fixed, dehydrated, and embedded while adherent to glass slides as indicated in the Materials and Methods. Thymocytes have been observed to bind to all of the free, nonglass-adherent surfaces of the macrophages. Sections cut in a plane tangential to the free macrophage surface cause thymocytes bound to this surface to appear to be completely surrounded by macrophage cytoplasm as in this photomicrograph. Serial sections show such thymocytes to be external to the macrophage. Magnification × 6,500.

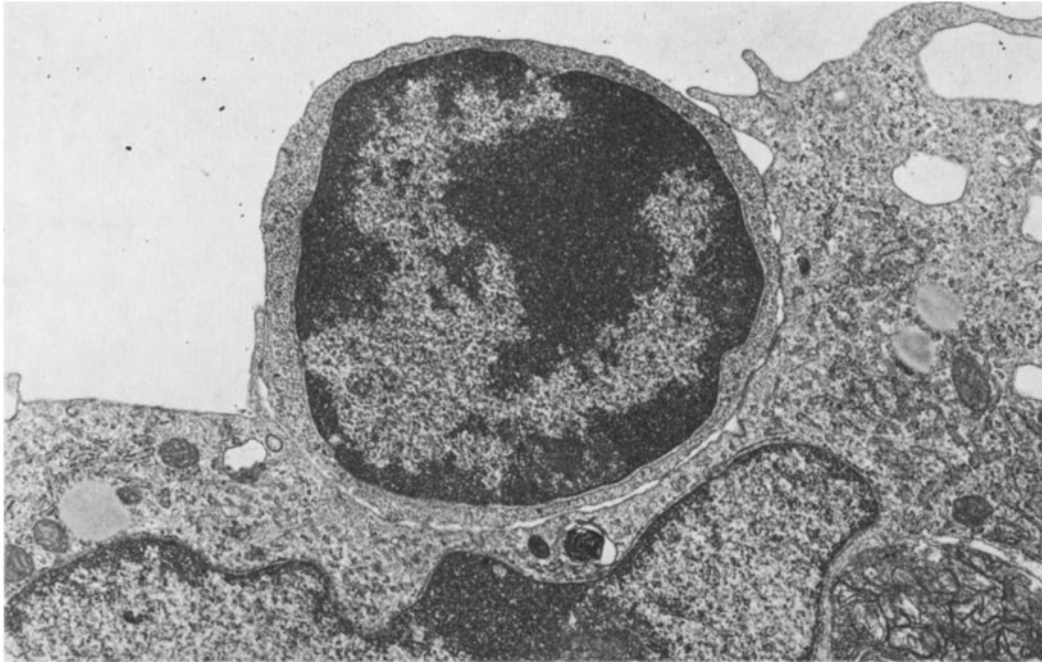


FIG. 8. Macrophage-thymocyte-binding. Broad areas of close contact were observed without evidence of cytoplasmic bridging. Magnification × 14,000.

of supporting antigen-mediated lymphocyte blast transformation than monocytes (8).

Microcinematographic studies have revealed dynamic physical interactions between lymphocytes and macrophages in vitro (1–3, 11). Our studies indicate that during these interactions a bond is established between the cells which is firm enough to resist rather vigorous washing procedures. Despite the strength of this bond, no ultrastructural specialization of the cellular architecture was observed in the areas of contiguity other than extremely broad areas of closely apposed plasma membrane. We have not seen convincing evidence of cytoplasmic bridging between cells. The kinetic nature of the cellular interactions studied in our system is suggested by the finding that prolongation of the incubation for up to 3 h did not result in increased thymocyte-binding above that observed after the first hour. The macrophages, however, had not been saturated with thymocytes, as evidenced by their ability to display increased binding when more thymocytes were presented, and no specialized subpopulation of "binding thymocytes" had been depleted. Thus, development of the observed plateau in macrophage-thymocyte interaction implies that an equilibrium between association and dissociation had been reached. The existence of such a steady-state is, furthermore, supported by observations made in our

laboratory utilizing radioisotope-labeled thymocytes.[4] In these studies, macrophage monolayers which had bound maximal numbers of thymocytes were observed to be able to exchange bound unlabeled thymocytes for isotopically labeled thymocytes without changing the total number of thymocytes bound.

Investigation into the nature of the cells capable of participating in this cellular association revealed that, of the glass-adherent cell types studied, macrophages of various origins were more apt to bind thymocytes than either polymorphonuclear leukocytes or fibroblasts. The significance of the heterogeneity observed among the various macrophages is unclear, but a variety of other functional and metabolic differences between macrophage types are known (22, 23, 30). Despite differences in the degree of binding observed, the assumption seems justified that the ability to physically interact with lymphocytes is a characteristic which distinguishes macrophages from other glass-adherent cells. Studies utilizing various nonglass-adherent cell types revealed that thymocytes as well as lymph node lymphocytes are bound to macrophage monolayers in a quantitatively reproducible fashion. In other studies,[5] peritoneal exudate lymphocytes (25) were found to bind to macrophage monolayers in much the same manner as lymph node lymphocytes, while mouse thymocytes demonstrated no significant binding to guinea pig macrophages. The decreased binding of L_2C cells as well as the lack of binding by guinea pig RBC or mouse thymocytes demonstrates specificity for the nonglass-adherent member of this cellular interaction. Thus, there is specificity for each of the participant cell types, suggesting the existence of a unique cellular recognition mechanism.

A previous report has noted the selective removal of immunoglobulin-bearing lymphocytes from a complex mixture of mouse lymphoid cells by plating over macrophage monolayers (31). The suggestion was made that a selective binding of the immunoglobulin-bearing lymphocytes to macrophages occurred by virtue of the macrophage receptor for immunoglobulin. However, in our studies, L_2C leukemia cells, known to bear γ_2-immunoglobulin on their surfaces (27), bound to macrophages less well than either thymocytes or column-purified lymph node lymphocytes. Further studies in our laboratory[3] using rabbit antibody directed against guinea pig immunoglobulin coupled to horseradish peroxidase to identify immunoglobulin-bearing lymphocytes (32) demonstrated that normal immunoglobulin-bearing lymphocytes obtained from lymph nodes are capable of, but not more likely to bind to macrophages than cells devoid of detectable surface immunoglobulin. Furthermore, modulation of immunoglobulin from the surface of immunoglobulin-bearing cells (32) did not diminish their binding and no competitive inhibition of binding by excess immunoglobulin could be demonstrated. These observations together with the data obtained using L_2C cells suggest that lymphocyte binding to macrophages does not involve the surface immunoglobulin of these cells.

The ability of heat-killed thymocytes to bind to macrophages indicates that

[4] Lipsky, P. E., J. T. Blake, and A. S. Rosenthal. 1973. Manuscript in preparation.

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

the thymocyte's role in this interaction is a passive one which does not require active cell metabolism. This is supported by the observation that thymocytes which had been treated with the irreversible metabolic inhibitor sodium iodoacetate were fully capable of being bound by macrophages. Although the thymocyte is a passive participant in the initial development of this intercellular bond, an active role for the nonglass-adherent cells in other aspects of interactions with macrophages has not been excluded. Thus, the more active movement of the lymphocyte, as revealed by microcinematographic studies (1–3, 11) may be important in bringing lymphocytes and macrophages into proximity so that specific-binding can occur. The termination of binding may, also, in part, be determined by an active function of the lymphocyte. In this regard, the increased net binding of heat-killed thymocytes by viable macrophages may reflect the loss of active thymocyte dissociation. The inability of heat-killed macrophages to bind thymocytes demonstrates the dependence of this phenomenon on the presence of intact, viable macrophages. The possibility that the macrophage component of the recognition mechanism was destroyed by heating was not specifically explored. The evidence, however, that iodoacetate-treated macrophages were incapable of participating in the interaction indicates that active macrophage metabolism was necessary for macrophage-lymphocyte interaction to occur.

The studies utilizing inhibitors, enzymes, and other experimental manipulations were designed to describe the characteristics of the binding of thymocytes to macrophages. The recognition mechanism mediating this binding has many of the features of a macrophage "receptor" for lymphocytes whose characteristics can be compared to those of other known macrophage receptors. It can be differentiated from the macrophage receptor for immunoglobulin (7S) coated red cells by virtue of its temperature and trypsin sensitivity, its requirement for divalent cations and its lack of competitive inhibition by excess immunoglobulin (33–35). It differs from the receptor demonstrated on mouse macrophages for homologous 19S immunoglobulin-coated red cells by virtue of its trypsin sensitivity (36). Its Ca^{++} rather than Mg^{++} dependence as well as its suceptibility to sodium azide define it as different from the receptor for antibody and complement-coated red cells (34). It can be distinguished from the "particulate" receptor as exemplified by the binding of aldehyde-treated RBC by virtue of its dependence on divalent cations (35). Thus, it is likely that the binding of lymphocytes by macrophages is accomplished by a different receptor mechanism than the ones which have been previously described to mediate the binding of particles to macrophages. Furthermore, it is unlikely that this binding occurs by virtue of immunoglobulin and/or complement associated with the thymocytes or lymphocytes.

In vivo, lymphocytes have been observed to be heterogeneous in regard to their migratory patterns (37). It is possible that these migration properties are related to the differential ability of various lymphocyte populations to develop

bonds with the indigenous macrophages of the lymphoid organs. Support for this concept comes from studies using enzyme-treated cells. Neuraminidase treatment of thymocytes markedly enhances their binding to macrophages in vitro. Neuraminidase treatment of thymocytes has also been noted to alter their subsequent in vivo migration (38). On the other hand, trypsin treatment of the thymocytes, which has less effect on their in vitro binding to macrophages in our assay has correspondingly little effect on in vivo migration.

Trephocytic functions of macrophages may be facilitated by the cellular proximity which results from this interaction. Thus, macrophages, but not fibroblasts or macrophage culture supernatants, could promote improved lymhpocyte viability in vitro (20). Likewise, the maturation and differentiation of functionally immature thymocytes occurred in vitro when these cells were cultured on supporting cell monolayers consisting of either thymic epithelial cells or splenic macrophages (21). The inability of soluble factors to substitute for monolayers themselves suggested that direct cell to cell contact was required, perhaps to facilitate the transfer of needed nutrients. Indeed, transmission of isotopically-labeled materials from macrophages to lymphocytes during periods of physical contiguity has been observed (13).

In the guinea pig (39), as in other species (5, 6, 40, 42), cellular cooperation between macrophages and immunocompetent lymphocytes has been demonstrated during the in vitro expression of antigen responsiveness. Antigen bound to macrophages triggers immunocompetent cells very efficiently as evidenced by the ability of antigen pulsed macrophages to stimulate immune lymphoid cells to the same degree as larger concentrations of free antigen (6, 8, 26, 40, 41). Supernatants from cultures of macrophages which have been exposed to antigen cannot induce antigen-specific lymphocyte stimulation (26, 39) suggesting that the required cellular cooperation involves direct cell to cell contact. The importance of cell contact is further emphasized by the observation that during in vitro immune induction, the vast majority of antibody-forming cells were found in clusters of lymphocytes and macrophages while disruption of these cell clusters blocked antibody formation (7, 19). When [H^3]thymidine incorporation was used as a marker for antigen-specific lymphocyte proliferation in vitro, it was observed that cluster formation preceded [H^3]thymidine incorporation (12), and that the majority of [H^3]thymidine-labeled cells were found physically associated with antigen-containing macrophages (8, 12) during the early period of proliferation.

Thus, much evidence exists that lymphocytes must physically interact with macrophages in order to manifest an antigen-dependent immune response in vitro. Since macrophage-lymphocyte interaction can occur in the absence of relevant antigen, it is reasonable to speculate that an early event in the immune response may involve antigen-independent reversible binding of lymphocytes to macrophages by means of a specific cellular recognition mechanism. Only when the macrophages had previously taken up antigen and retained it in

immunogenic form would this initial binding step serve to select specifically immunocompetent lymphocytes and promote their proliferation and/or differentiation.

SUMMARY

The nature of the physical interaction between guinea pig non-glass-adherent lymphoid cells and syngeneic macrophages in vitro was investigated. This cellular interaction was found to require the presence of metabolically intact macrophages but neither serum nor antigen. Peritoneal, splenic, or alveolar macrophages were significantly more capable of interacting with thymocytes than either polymorphonuclear leukocytes or fibroblasts. The role of the non-glass-adherent cell was passive in that heat-killed or metabolically poisoned thymocytes were bound by normal macrophages. Two normal lymphoid cell populations, thymocytes, and lymph node lymphocytes, were bound to macrophages in significantly larger numbers than either L_2C leukemia cells or erythrocytes. Thus, specificity for each of the participant cell types was demonstrated. These data indicate that macrophages possess a unique ability to recognize and bind lymphocytes and thymocytes by a mechanism which is distinguishable from other known macrophage receptors.

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