

THE SPECIFIC BINDING OF *LISTERIA MONOCYTOGENES*- IMMUNE T LYMPHOCYTES TO MACROPHAGES

I. Quantitation and Role of H-2 Gene Products*

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During the past decade, numerous studies have firmly established the role of immune response (Ir) genes in the control of immune responses (1, 2). This control, demonstrated by in vivo and in vitro assays of both humoral and cell-mediated immune responses, has been examined most incisively by antibody formation (3, 4), delayed-type hypersensitivity (5, 6), and T-cell proliferative responses (7, 8) to polypeptide antigens of limited heterogeneity.

Studies of the immune response to complex antigens such as the intracellular pathogen *Listeria monocytogenes* have implied a role for Ir gene products, even though animals completely unresponsive to *L. monocytogenes* remain unidentified. The inferred involvement of Ir genes in the control of *L. monocytogenes* reactivity derived from observations that adoptive transfer of immunity to *L. monocytogenes* occurs optimally when the donor of immune T cells and the host are homologous at the I region of the H-2 gene complex (9). Complementary studies on the function of *L. monocytogenes*-specific T cells in vitro have also revealed that antigen-specific, T-cell-induced macrophage activation occurs best when T cells and macrophages are derived from I region (I-A) homologous mice (10-12). Such macrophage activation, believed to be of a physiological importance in the efficient ingestion and destruction of bacteria, is manifested in vitro by a number of morphologic, biochemical, and functional stages (13, 14). Two of these parameters, readily quantifiable in vitro, are the acquisition of macrophage cytotoxic activity and an enhanced secretion of a protein mitogenic for thymocytes (10-12). The expression of these parameters of T-cell-macrophage function requires a population of Ia antigen-bearing macrophages, and such induction is blocked by antibodies directed against Ia antigens (11, 12). The importance of T-cell-macrophage contact in generating T-cell-macrophage activation is suggested by the lack of activation when the two cell types are separated by cell-impermeable membranes (10).

In view of the hypothesis that T cells recognize antigen in association with Ir gene products (15), the studies presented here were aimed at a more careful evaluation of the role of T-cell-macrophage contact in T-cell activation and a determination of whether Ir gene products dictate the antigen-specific physical interaction between T cells and macrophages. To this end, a system was developed to allow the testing of the correlation between direct measurements of activation with a quantitation of specific

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T-cell-macrophage binding. The results presented here are consistent with the hypothesis that T-cell-macrophage binding is one initial, and determining, event in Ir gene control of immune reactivity.

Materials and Methods

Mice. In the experiments presented here, male or female mice were used at 8- to 12-wk of age. A/St mice were purchased from West Seneca Laboratories, Buffalo, N. Y.). A/J, C57BL/6, BALB/c, C3H, and (C57BL/6 × A)F₁ (B6AF₁) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Bacteria. The preparation of *L. monocytogenes* has been described previously (10). Briefly, aliquots of *L. monocytogenes* organisms, stored at -20°C, were incubated (20 h, 37°C) in brain-heart infusion broth (Difco Laboratories, Detroit, Mich.), washed extensively in phosphate-buffered saline, and their number estimated by measurements of turbidity later confirmed by colony enumeration. Bacteria, thus treated, were used immediately to infect animals as described below or were heat killed (63°C, 1.5 h) for use in vitro.

L. monocytogenes-immune T Cells. Mice were injected i.p. with $1-5 \times 10^4$ live *L. monocytogenes* organisms (LD₅₀: 2×10^5 bacteria). 7 d later, an i.p. injection of 1.5 ml of a sterile 10% solution (wt/vol) of proteose peptone (Difco Laboratories) was administered, and the peritoneal exudate cells harvested 3 d later (i.e., 10 d after initial infection). Peritoneal-exudate-cell harvesting was accomplished by peritoneal lavage with cold Hanks' balanced salt solution (Microbiological Associates, Walkersville, Md.) containing 2.5% fetal calf serum (FCS),¹ 10 mM Hepes buffer (Microbiological Associates), and heparin (10 U/ml). The cells were washed and then resuspended in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) containing 5% FCS, 10 mM Hepes buffer, 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml). All subsequent tissue culture was performed with this medium. T-cell enrichment was accomplished by removal of cells adherent to tissue-culture dishes and nylon wool as described previously (10-12). Peritoneal exudate lymphocytes, thus prepared, were >98% Thy-1.2 positive, as judged by immunofluorescence, and contained <0.5% macrophages, as estimated by morphology and phagocytosis of latex particles (10-12).

Macrophages. In all of the experiments presented here, peptone-elicited peritoneal exudate cells from normal mice were used as a source of macrophages. Such cells were obtained by peritoneal lavage (as described above for *L. monocytogenes-immune T cells*) 3 d after i.p. injection of 1.5 ml of proteose peptone (10% solution). Peritoneal cells containing ~70-80% macrophages were plated on tissue-culture vessels (1-2 h, 37°C) and then washed extensively to remove nonadherent cells. Greater than 98% of the remaining adherent cells displayed morphological and receptor characteristics of macrophages.

Assay of Mitogenic Protein Production. The system used to quantitate T-cell-induced mitogenic protein production, apparently by macrophages, represents an adaptation of that previously described by Farr, et al. (10-12). Flat-bottomed multi-well tissue-culture plates (area per well: 28 cm²) (Linbro Chemical Co., Hamden, Conn.) were used as culture vessels and RPMI-1640, supplemented with 5% FCS, 2 mM L-glutamine, 10 mM HEPES, penicillin (50 U/ml), and streptomycin (50 µg/ml) as culture media.

Peptone-elicited peritoneal exudate cells (3×10^5 per well) were incubated for 1-2 h at 37°C and then the nonadherent cells removed by vigorous pipetting and washing. Heat-killed *L. monocytogenes* organisms (0.2 ml per well, 10^7 bacteria per ml) were then incubated (1 h, 37°C) with the adherent cells (i.e., macrophages). After incubation, the *L. monocytogenes* that were not phagocytized were removed by vigorous washing. Macrophages, thus treated, will be referred to as *L. monocytogenes-pulsed macrophages*. Varying numbers of *L. monocytogenes-immune T cells* (10^3-10^5 cells per well) were added to the macrophage monolayers and coincubated in a total vol of 0.2 ml for 20-24 h at 37°C. After incubation, the microtiter plates were centrifuged (800 rpm, 5 min), and 0.1 ml of cell-free supernate from each well was transferred to another well of a round-bottomed multi-well tissue-culture plate (Linbro Chemical Co.). Thymocytes (10^6 per well), prepared from A/St mice, were then added to the culture supernates and

¹ Abbreviations used in this paper: FCS, fetal calf serum; NMS, normal mouse serum.

incubated (3 d, 37°C) in a total vol of 200 μ l per well. Tritium-labeled thymidine (0.2 μ Ci per well) (2 Ci/mM, New England Nuclear, Boston, Mass.) was present during the last 18 h of culture. The amount of 3 H-label incorporated into trichloroacetic acid-insoluble material was measured by standard procedures.

Thymocytes cultured alone or those cultured with supernates derived from culture of T cells in the absence of macrophages routinely yielded 50–150 cpm. Fluids from macrophage cultures in the absence of T cells or *L. monocytogenes* gave ~200–500 cpm. Supernates from *L. monocytogenes*-pulsed macrophages showed ~300–600 cpm, whereas those derived from a coculture of *L. monocytogenes*-pulsed macrophages and 10^5 *L. monocytogenes*-immune T cells routinely produced [3 H]thymidine incorporation of 10,000–20,000 cpm. Clearly, T-cell-dependent mitogenic protein production required both specifically immune T cells and macrophages pulsed with *L. monocytogenes* (11).

Duplicate or triplicate cultures seldom varied by $> \pm 5\%$. For the experiments described in the text, the term "significance" is used only to indicate statistical significance ($P \leq 0.05$). Data are expressed as the T-cell-dependent counts per minute (i.e., Δ counts per minute equals counts per minute produced by supernates from cocultures of *L. monocytogenes*-pulsed monolayers and *L. monocytogenes*-specific T cells minus the counts per minute produced by culture supernates from *L. monocytogenes*-pulsed macrophages in the absence of *L. monocytogenes*-specific T cells).

Assay of Macrophage-mediated Cytotoxicity. The assessment of T-cell-induced macrophage-mediated cytotoxicity involves a 24-h T-cell-dependent induction period followed by a 16- to 24-h T-cell-independent macrophage-mediated cytotoxicity phase (12). Previous studies had clearly indicated that the killing of tumor cells, the cytotoxicity phase of the reaction, was mediated by the activated macrophages and did not involve any contribution from the immune T-cell inoculum (12). The induction of macrophages to a cytotoxic state was performed in a manner identical to that described above for the generation of mitogenic protein. After the 24-h coinubation of T cells and *L. monocytogenes*-pulsed macrophages, the supernate was aspirated, and then 0.2 ml of medium per well containing 2×10^4 51 Cr-labeled P815 tumor cells were added to the cultured macrophages (12). After a 20-h incubation at 37°C, 0.1 ml of cell-free supernate was assayed for 51 Cr content.

The percentage of specific cytolysis was calculated as follows: Let E equal the 51 Cr released by target cells in the presence of *L. monocytogenes*-pulsed macrophages precultured with *L. monocytogenes*-immune T cells. Let C equal the 51 Cr released in the presence of *L. monocytogenes*-pulsed macrophages precultured without *L. monocytogenes*-immune T cells. Let T equal the 51 Cr released when target cells were subjected to one cycle of freezing and thawing. Therefore:

$$\text{the percentage of specific cytolysis} = \frac{E - C}{T - C} \times 100.$$

The value of C was usually 25–35% of the value for T and was similar to the values of 51 Cr release for target cells alone or targets in the presence of macrophages precultured alone. Values for the percentage specific cytolysis seldom varied by $> \pm 5\%$. Like the T-cell-dependent production of mitogenic protein, T-cell-induced macrophage-mediated cytotoxicity was antigen specific (12). In this system, macrophages with or without a *L. monocytogenes* pulse did not kill 51 Cr-labeled P815 cells if the macrophages were not incubated with T cells (12). For simplicity, the only cytotoxicity results shown are those involving macrophage activation in the presence of *L. monocytogenes* and immune T cells.

Quantitation of T-Cell-Macrophage Binding (Fig. 1). Confluent macrophage monolayers were prepared in 16-mm Diam tissue culture wells (Linbro Chemical Co.) by adding 1.2×10^6 peptone-elicited peritoneal exudate cells to each well and then culturing at 37°C for 2 h. Nonadherent cells were then removed by extensive washing. The macrophage monolayers thus formed (binding macrophages [BINDING MACS, Fig. 1]) were incubated (1 h, 37°C), with heat-killed *L. monocytogenes* (0.5 ml per well, $1-2 \times 10^7$ bacteria per ml) and then washed extensively to remove unbound bacteria. Control monolayers were incubated with medium alone. *L. monocytogenes*-immune T cells (10^6 per monolayer) were added, the tissue-culture dishes centrifuged (50 g, 4 min, 4°C), and then incubated for 1 h at 37°C (unless stated otherwise). After gentle agitation of the plates, the nonadherent T cells were collected, counted, and then their macrophage-activating function was assessed on a new set of macrophage monolayers,

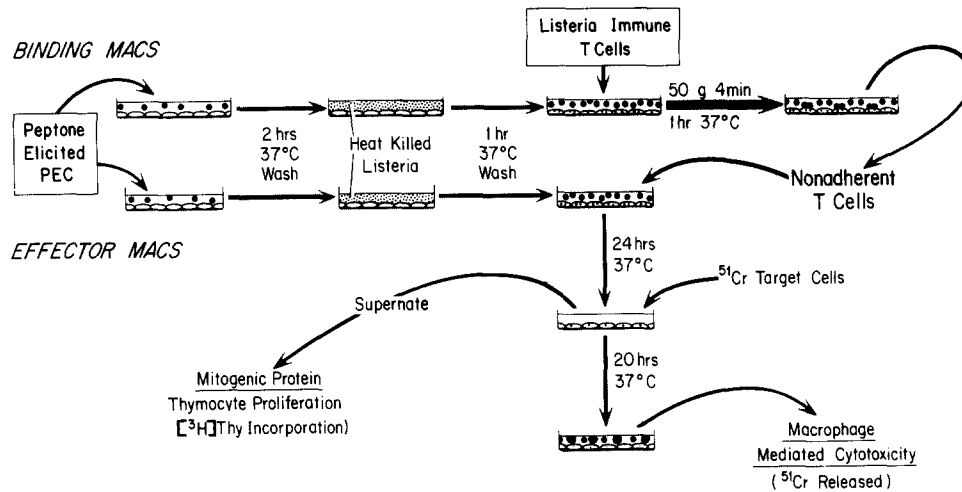


FIG. 1. Quantitation of T-cell-macrophage binding. Schematic representation of the protocol used to quantitate T-cell-macrophage binding by the functional depletion of *L. monocytogenes*-specific T cells in the cells nonadherent to *L. monocytogenes*-pulsed macrophage monolayers. PEC, peritoneal exudate cells; BINDING MACS, binding macrophages; EFFECTOR MACS, effector macrophages.

these adherent cells are referred to as effector macrophages. Such activation was monitored by the mitogenic-protein- and/or cytotoxicity-readout systems described above, both of which gave similar results.

The decrease in activity of T cells nonadherent to macrophage monolayers was usually taken as a measure of T-cell-macrophage binding.

The percentage of binding was calculated as follows: Let X equal the activity of T cells nonadherent to wells containing no macrophages. Let Y equal activity of T cells nonadherent to macrophage monolayers. Then:

$$\text{the percentage of binding} = \frac{X - Y}{X} \times 100.$$

The value of X was empirically determined to be not significantly different from that of untreated T cells. Thus, both X and untreated T cells will be termed unfractionated.

The percentage of specific binding was calculated as follows: Let A equal activity of T cells nonadherent to macrophage monolayers (no *L. monocytogenes*). Let B equal activity of T cells nonadherent to *L. monocytogenes*-pulsed macrophage monolayers. Then:

$$\text{the percentage of specific binding} = \frac{A - B}{A} \times 100.$$

In both cases, T-cell activity was expressed as the percentage of specific cytolysis for the macrophage cytidal readout system and as Δ counts per minute for the mitogenic protein assay.

In some experiments the percentage of specific binding was calculated as above on the basis of the number, rather than the activity, of T cells recovered from the macrophage monolayers.

Antiserum. Anti-H-2 serum was produced by multiple weekly injections of B10.A spleen and lymph node cells i.p. into B10.D2 recipients. Anti-Ia (A.TH-anti-A.TL) antiserum was generously provided by Dr. David Sachs of the National Institutes of Health, Bethesda, Md. and Dr. Martin Dorf of the Department of Pathology, Harvard Medical School, Boston, Mass. These reagents were prepared and characterized by standard serological methods. In the blocking studies to be discussed, a dilution of antiserum which produced optimal complement-mediated lysis of appropriate macrophages was employed.

TABLE I
Conditions Required to Detect T-Cell-Macrophage Binding

Binding substrate*	Condition‡	Percentage of T-cell-induced, macrophage-mediated cytotoxicity§	Percentage of specific binding
		%	%
Macrophage	1 g, 4°C	27.7 ± 1.0	—
Macrophage + 10 ⁷ <i>L. monocytogenes</i>		35.3 ± 2.3	0
Macrophage + 10 ⁹ <i>L. monocytogenes</i>		37.1 ± 0.5	0
Macrophage	1 g, 37°C	29.4 ± 0.7	—
Macrophage + 10 ⁷ <i>L. monocytogenes</i>		21.9 ± 2.0	25.5
Macrophage + 10 ⁹ <i>L. monocytogenes</i>		11.4 ± 1.8	61.2
Macrophage	200 g, 4°C	37.0 ± 0.6	—
Macrophage + 10 ⁷ <i>L. monocytogenes</i>		21.9 ± 3.4	40.8
Macrophage + 10 ⁹ <i>L. monocytogenes</i>		17.8 ± 0.8	51.8
Macrophage	200 g, 37°C	34.3 ± 1.9	—
Macrophage + 10 ⁷ <i>L. monocytogenes</i>		0.3 ± 0.1	99.1
Macrophage + 10 ⁹ <i>L. monocytogenes</i>		13.1 ± 2.2	61.8
Plastic	1 g, 4°C	26.8 ± 2.1	—

L. monocytogenes-immune T cells from A/St mice were fractionated on the binding substrate as indicated and then tested for their ability to induce cytotoxic activity in syngeneic macrophages. The percentage of specific binding was calculated as described in Materials and Methods.

* Macrophage monolayers from A/St peritoneal exudate cells were incubated (2 h, 37°C) with 10⁷ or 10⁹ heat-killed *L. monocytogenes* per ml. Unbound *L. monocytogenes* was removed by washing before addition of T cells.

‡ After the addition of *L. monocytogenes*-immune T cells to the binding substrate, the plates were centrifuged (200 g, 2 min, 4°C) or incubated at 4°C for 2 min (1 g) and the incubated for 1 h at the temperature indicated. The nonadherent T cells were recovered and then subjected to another identical binding condition. T cells recovered after the second binding cycle were then tested for function.

§ T-cell-induced macrophage-mediated cytotoxicity was assessed as described in Materials and Methods for 1 × 10⁶ fractionated T cells and expressed as the percentage of specific cytolysis plus or minus SD. The spontaneous release of ⁵¹Cr in this experiment was 25%. P815 cells incubated with macrophages pulsed with *L. monocytogenes* did not show specific release of ⁵¹Cr. The value for T was 10,252 cpm.

|| T cells were fractionated on tissue culture plates containing no macrophages (plastic). The activity of such cells was identical to the activity of T cells fractionated on macrophage monolayers (no *L. monocytogenes*) at 1 g but slightly lower than those T cells similarly fractionated at 200 g. This slight difference, apparently unique to conditions employing higher centrifugal forces (e.g., 200 g rather than 50 g), was perhaps a result of the selective removal of non-*L. monocytogenes*-specific T cells.

Results

Requirements for Detecting T-Cell-Macrophage Binding. Initial experiments were performed to determine the conditions under which T-cell-macrophage binding could be detected; the depletion of specific T-cell function in the population of cells nonadherent to macrophage monolayers was taken as a measure of such binding. The binding conditions investigated included the following parameters: (a) the amount of heat-killed *L. monocytogenes* used to pulse the binding macrophage; (b) the gravitational force used to initiate T-cell-macrophage contact; (c) the temperature at which the binding reaction was conducted; and (d) the number of cycles of adherence performed

before assessment of T-cell function and the duration of T-cell-macrophage exposure. The experiment depicted in Table I represents one example of this approach.

Macrophages from A/St mice were used as the binding substrate (binding macrophages, Table I) and were pulsed with either 10^7 or 10^9 heat-killed *L. monocytogenes* organisms per ml for 2 h at 37°C. Syngeneic *L. monocytogenes*-immune T cells (10^6 per well) were added, the plates centrifuged (200 g, 2 min, 4°C), and then incubated at either 4°C or 37°C. Some binding reactions were incubated without prior centrifugation (1 g). After incubation for 1 h at the temperature indicated, the non-adherent T cells were recovered and then placed on another set of identical macrophage-binding substrates and the same cycle of initiation and incubation repeated. After the second cycle of adherence, the nonadherent T cells were collected, washed, counted, and then tested for their function in inducing cytotoxic activity in a new set of syngeneic macrophage monolayers (effector macrophages, Table I).

As can be seen in Table I, the activity of T cells fractionated on macrophage monolayers that received no *L. monocytogenes* pretreatment was no less than the activity of T cells fractionated in parallel on tissue-culture wells containing no macrophages (plastic, Table I). Significant depletion of T-cell activity was observed when the macrophage-binding monolayers were pretreated with heat-killed *L. monocytogenes* (Macrophage + *L. monocytogenes*, Table I) and when T-cell-macrophage contact occurred under certain conditions. Although no specific binding was observed when contact was initiated at 1 g and the binding incubation conducted at 4°C, significant binding occurred when such initiation of contact was followed by incubation at 37°C. The binding reactions initiated by centrifugation were clearly more productive than those initiated without centrifugation. In fact, specific binding did occur at 4°C (200 g, 4°C), albeit, to a much lesser extent than at 37°C. Clearly, with macrophages pulsed with 10^7 *L. monocytogenes* per ml, the binding reaction initiated by centrifugation and then followed by incubation at 37°C was most effective in producing virtually complete depletion of T-cell function (99% specific binding). In other experiments of this type, conducted under conditions determined to be optimal, as in Table I, good specific binding (>40%) could be detected with binding macrophages pretreated for only 1 h (37°C) with as little as 10^5 to 10^6 heat-killed *L. monocytogenes* organisms and with a single cycle of adherence of T cells to macrophages.

Experiments were performed to analyze the kinetics of T-cell-macrophage binding. Results of one experiment employing *L. monocytogenes*-immune T cells and macrophages from A/St mice using the induction of macrophage-mediated cytotoxicity as the readout system for *L. monocytogenes*-specific T-cell function are shown in Figs. 2-4. Fig. 2 shows the activity of *L. monocytogenes*-immune T cells fractionated on either macrophage monolayers receiving no *L. monocytogenes* pretreatment (Fig. 2A, MACROPHAGE) or *L. monocytogenes*-pulsed macrophage monolayers (Fig. 2B, MACROPHAGE + LM). As can be seen, *L. monocytogenes*-immune T cells exposed to macrophage monolayers for 120 min showed activity differing marginally from that of unfractionated T cells, whereas those exposed for 5 min showed significantly less activity (i.e., some antigen-independent binding took place). In contrast, the activity of T cells nonadherent to *L. monocytogenes*-pulsed macrophages (Fig. 2B, MACROPHAGE + LM) progressively declined with increasing time of T-cell-macrophage exposure. These results, expressed as the percentage of binding, are shown in Fig. 3 for the activity of 10^5 T cells (Fig. 3A) and 3.3×10^4 T cells (Fig. 3B). As can be

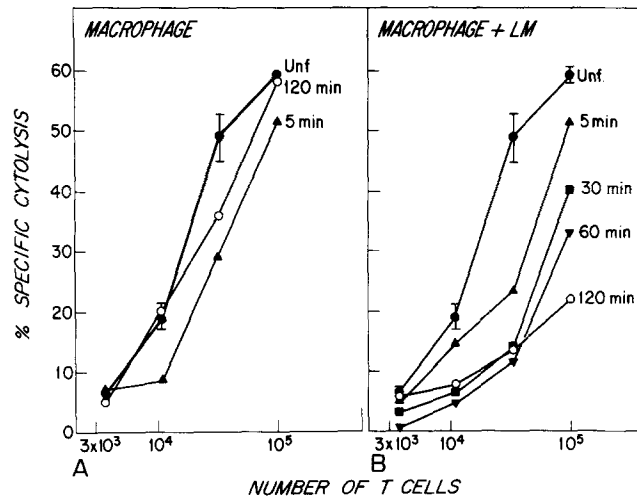


FIG. 2. The depletion of specific T-cell activity is shown with different binding-reaction times. The experiment was performed exactly as described in Materials and Methods except that *L. monocytogenes*-immune T cells were added to binding macrophages at various times after medium pretreatment (A) (MACROPHAGE) or *L. monocytogenes* pretreatment (B) (MACROPHAGE + LM) and then recovered at the same time such that the time of T-cell-macrophage exposure was varied as indicated. The activity of various numbers of fractionated T cells, as measured by the induction of macrophage-mediated cytotoxicity (% SPECIFIC CYTOLYSIS) is shown. Unfractionated T cells (Unf) represents cells exposed to wells containing no macrophages for 5 min and 120 min, their activity being given as the mean \pm the range of the two unfractionated populations. Results of activities of T cells binding to unpulsed macrophages (A) at 30 and 60 min were similar to 120 min and are not shown.

seen, antigen-independent T-cell macrophage binding (Fig. 3A, Mac) occurred quickly and then declined, whereas antigen-dependent binding (Mac + LM) increased with time.

Fig. 4 shows the plot of the percentage of specific binding of the results of Figs. 2 and 3 as a function of time. When small numbers of T cells were used in the readout system (1.1×10^4 , 3.3×10^4), significant specific binding was detected within 5 min and rose to completion by 60 min. In contrast, the functional assessment of a larger number of fractionated T cells (1×10^5) yielded values for the percentage of specific binding of lesser magnitude and thus seemingly slower kinetics.

This data, together with data from other experiments not shown, made it clear that antigen-independent binding could be optimally detected by short, multiple binding cycles, whereas antigen-dependent binding was most striking when a single but longer (60- to 120-min) binding cycle was performed. In subsequent experiments presented here, a 1- to 2-h binding reaction at 37°C was employed, and the depletion of specific T-cell function in the nonadherent cells was used as a measure of specific binding.

The percentage of specific binding, as measured by functional depletion with syngeneic combinations of cells, was estimated in 19 different experiments to be $72.3 \pm 4.0\%$ (mean \pm SEM). *L. monocytogenes*-specific binding could also be evidenced by the differential recoveries of T cells from the macrophage monolayers. Routinely, 50–60% of the total T cells added were recovered from the macrophage monolayers ($55.9 \pm 3.0\%$ SEM, $n = 14$), whereas 40–50% were recovered from macrophage monolayers pulsed with *L. monocytogenes* ($44.8 \pm 3.3\%$ SEM, $n = 14$). Thus, the percentage of

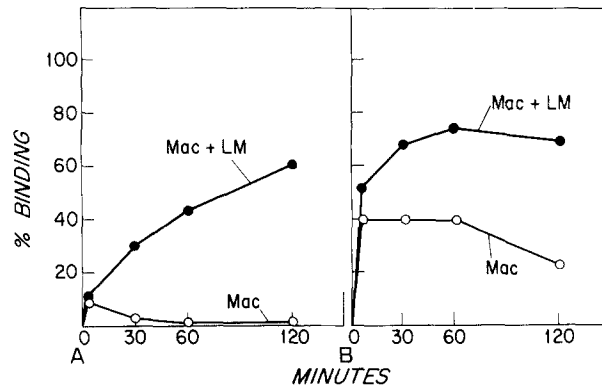


FIG. 3. The kinetics of the binding of specific T cells to macrophages (Mac) and *L. monocytogenes*-pulsed macrophages (Mac + LM) is given as the percentage binding (calculated as described in Materials and Methods) using the data illustrated in Fig. 2 at 10^5 (A) and 3.3×10^4 (B) fractionated cells.

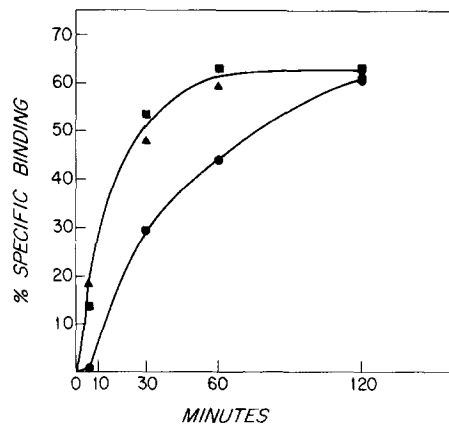


FIG. 4. The kinetics of *L. monocytogenes*-specific T-cell-macrophage binding are illustrated as the percentage of specific binding calculated (as described in Materials and Methods) with the indicated number (\bullet , 1×10^5 ; \blacktriangle , 3.3×10^4 ; \blacksquare , 1.1×10^4) of T cells in the macrophage-mediated cytotoxicity assay system shown in Fig. 2.

specific binding calculated on the basis of number of T cells recovered was $20.6 \pm 3.8\%$ (mean \pm SEM, $n = 14$).

Limited attempts have been made to recover the *L. monocytogenes*-immune T cells adherent to the *L. monocytogenes*-pulsed macrophages. Experiments along these lines are very much in progress and will be presented in detail in a further communication. In the preliminary experiments performed so far, the macrophage-adherent T cells, when recovered by vigorous agitation 16 h after the binding reaction was performed, when assayed, showed a two- to fourfold-greater activity per cell than that of unfractionated T cells. For example, unfractionated T cells induced a 19% specific macrophage-mediated cytolysis, whereas an equal number of T cells recovered from the macrophages gave a 55.3% specific cytolysis. The physical retention of *L. monocytogenes*-specific T cells by the macrophage monolayers could also be evidenced by the enhanced activation of the macrophage monolayers used as the binding substrate.

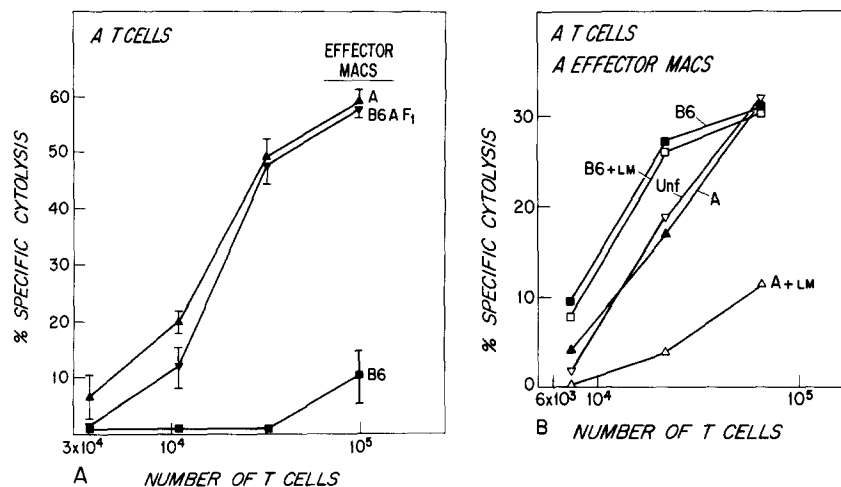


FIG. 5. (A) Varying numbers of *L. monocytogenes*-immune T cells prepared from A/St mice (A-T CELLS) were added to *L. monocytogenes*-pulsed (10^7 organisms per ml, 1 h, 37°C) peptone-elicited effector macrophages (EFFECTOR MACS) (3×10^6 peritoneal cells per well) derived from A/St (A), (C57BL/6 \times A) F_1 (B6AF $_1$), or C57BL/6 (B6) mice. Number of cells refers to the number of T cells added to each well. After incubation for 24 h at 37°C , the effector macrophages were washed, and then 2×10^4 ^{51}Cr -labeled P815 cells were added. The ^{51}Cr released after a 20-h incubation at 37°C was assessed and then used to calculate the percentage of specific cytolysis as described in Materials and Methods. Not plotted are the results of the *L. monocytogenes*-pulsed macrophages not cultured with T cells that did not kill P815 cells.

(B) *L. monocytogenes*-immune T cells from A/St mice (A-T CELLS) were fractionated on various macrophage monolayers as indicated, and different numbers of the nonadherent T cells were then tested for function with effector macrophages from syngeneic mice (A-EFFECTOR MACS). The binding reaction was performed exactly as described in Materials and Methods and as depicted in Fig. 1. B6, C57BL/6 macrophages; B6 + LM, B6 macrophages pulsed with *L. monocytogenes*; A, A/St macrophages; A + LM, A macrophages pulsed with *L. monocytogenes*; Unf, unfractionated.

Thus, the functional depletion of *L. monocytogenes*-specific T-cell function in macrophage nonadherent cells appears to be a reasonable measure of T-cell-macrophage binding.

The question of the specificity of binding of T cells to the *L. monocytogenes*-pulsed macrophages has been examined in a number of ways. Clearly, the T cells did not adhere nonspecifically to *L. monocytogenes*-pulsed macrophages as best illustrated in the genetic restriction experiments to be described next. In other experiments, the *L. monocytogenes*-immune T cells did not bind to macrophage monolayers pulsed with hemocyanin. When macrophages were pulsed with *Staphylococcus aureus*, there was some binding of *L. monocytogenes*-immune T cells: 20% specific binding compared to 50% for *L. monocytogenes*-pulsed macrophages. These results are in keeping with the documented serological (16) and cellular (12) cross-reactivity of both organisms.

Genetic Restrictions of T-Cell-Macrophage Binding. In attempts to determine whether T-cell-macrophage binding showed genetic restrictions similar to those observed with direct measurements of function (10–12), a series of experiments was performed with macrophages of differing genetic composition. Initial experiments to test the correlation of activation with binding function are shown in Fig. 5.

In keeping with previous observations (12), *L. monocytogenes*-immune T cells from A/St mice (Fig. 5A, A T CELLS) induced a cytotoxic activity when cultured for 24

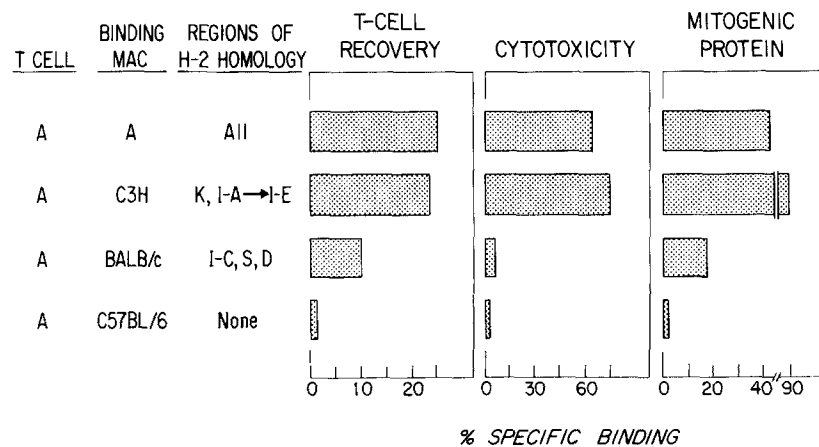


Fig. 6. *L. monocytogenes*-immune T cells from A/St mice (A-T CELLS) were fractionated on binding macrophages derived from the mouse strains indicated and then the percentage of specific binding estimated by: (a) the number of T cells recovered from *L. monocytogenes*-pulsed macrophages relative to untreated macrophage monolayers (T-CELL RECOVERY); (b) the activity of fractionated T cells in the induction of macrophage-mediated cytotoxicity (CYTOTOXICITY); and (c) the function of nonadherent T cells in mitogenic protein secretion (MITOGENIC PROTEIN). The latter two functional tests were performed with syngeneic (A) effector macrophages, and the percentage of specific binding was calculated (as described in Materials and Methods) for two different numbers of fractionated T cells (1.1×10^4 and 3.3×10^4); the mean percentage of specific binding of both is depicted.

h with syngeneic (Fig. 5A, A), and semi-syngeneic (B6AF₁) effector macrophages pulsed with *L. monocytogenes*, but were almost completely ineffective when cultured with allogeneic C57BL/6 macrophages (Fig. 5A, B6). Likewise, when *L. monocytogenes*-immune T cells from A/St mice (Fig. 5B, A T cells) were fractionated on syngeneic or allogeneic macrophage monolayers, only those T cells fractionated on syngeneic macrophages pulsed with *L. monocytogenes* (5B, A + LM) showed significant functional depletion indicating specific binding only to the syngeneic macrophages.

Previous studies of Farr, et al., have shown that to produce specific T-cell-induced macrophage activation, as measured both by the enhanced secretion of mitogenic protein and by the acquisition of macrophage cytotoxic function, the T cell and the macrophage had to share the I region of the H-2 gene complex (11, 12). Thus, a study was performed to test whether the specific T-cell-macrophage binding as measured here was similarly genetically restricted. Such an experiment is shown in Fig. 6.

L. monocytogenes-immune T cells from A/St mice (Fig. 6) were tested for their binding to the macrophages as indicated, and the percentage specific binding was assessed by three independent means: (a) the number of T cells recovered from the macrophage monolayers (Fig. 6, T-CELL RECOVERY); (b) the capacity of recovered T cells to induce cytotoxic activity in syngeneic macrophages (Fig. 6, CYTOTOXICITY); and (c) secretion of mitogenic protein (Fig. 6, MITOGENIC PROTEIN). As can be seen, substantial specific binding occurred with A and C3H binding macrophages, but little or no binding occurred with BALB/c or C57BL/6 macrophages. These results are consistent with the conclusion that the region of the H-2 gene complex dictating specific T-cell-macrophage binding lies within the K through I-E sub-region. Thus, the correlation of activation with binding function holds for such genetic restrictions.

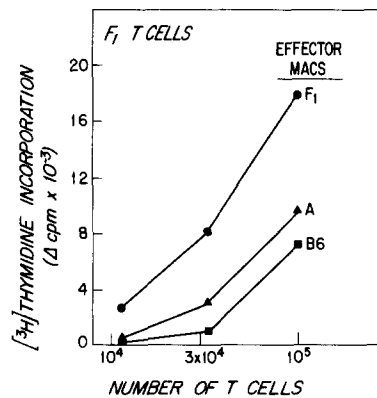


FIG. 7. Varying numbers of *L. monocytogenes*-immune T cells from B6AF₁ mice (F₁-T CELLS) were coincubated (20 h, 37°C) with *L. monocytogenes*-pulsed effector macrophages obtained from B6AF₁ (F₁), A/St (A), or C57BL/6 (B6) mice. Data derived from the mitogenic protein assay are shown. The amounts of mitogen secreted by cultures of macrophages pulsed with *L. monocytogenes* without T cells never exceeded 10^3 cpm.

In a further series of experiments, *L. monocytogenes*-immune T cells from B6AF₁ mice were employed to test for the possible clonal nature of genetic restrictions as suggested by previous studies (8, 17-19). When *L. monocytogenes*-immune T cells from B6AF₁ mice (Fig. 7, F₁ T CELLS) were tested for their ability to activate B6AF₁ (Fig. 7, F₁) and parental macrophages, the results shown in Fig. 7 were obtained using mitogenic protein as the readout system. Clearly, the magnitude of response by effector macrophages derived from either A/St or C57BL/6 parental mice (Fig. 7, A; B6) was less than one-half that observed with B6AF₁ effector macrophages, implying that at least two clones were operating in this system.

Direct evidence for the clonal nature of the response by B6AF₁ T cells was sought by fractionating B6AF₁ T cells on B6AF₁, A/St, and C57BL/6 binding macrophages and then testing the activity of the nonadherent T cells on B6AF₁, A/St, and C57BL/6 effector macrophages. The results of such an experiment using mitogenic protein as a readout are shown in Fig. 8. Those B6AF₁ T cells capable of activating B6AF₁ effector macrophages were depleted to great extent only when fractionated on *L. monocytogenes*-pulsed B6AF₁ binding macrophages (Fig. 8; F₁ Effectors, F₁ + LM). When B6AF₁ T cells were measured on A effector macrophages, significant depletion was apparent only following fractionation on *L. monocytogenes*-pulsed B6AF₁ and A/St binding macrophages. In contrast, the activity of B6AF₁ T cells, when measured on C57BL/6 effector macrophages, was found to be reduced only in the cells nonadherent to *L. monocytogenes*-pulsed B6AF₁ and C57BL/6-binding macrophages. In Fig. 9, the pooled data from two experiments of this type are given as percent specific binding (mean \pm range). At least two clones of B6AF₁-T cells are apparent.

In order to more definitively establish the role of H-2 gene products in specific T-cell-macrophage binding, an experiment similar to those described in Figs. 8 and 9 was performed. This time, an attempt was made to block specific binding with antibodies directed against the products of the K end of the H-2^a haplotype (B10.D2-anti-B10.A). The experiment depicted in Fig. 10 was performed as follows. Binding macrophages were pulsed with heat-killed *L. monocytogenes* for 1 hour at 37°C and then washed thoroughly. Control macrophages received no *L. monocytogenes* pretreat-

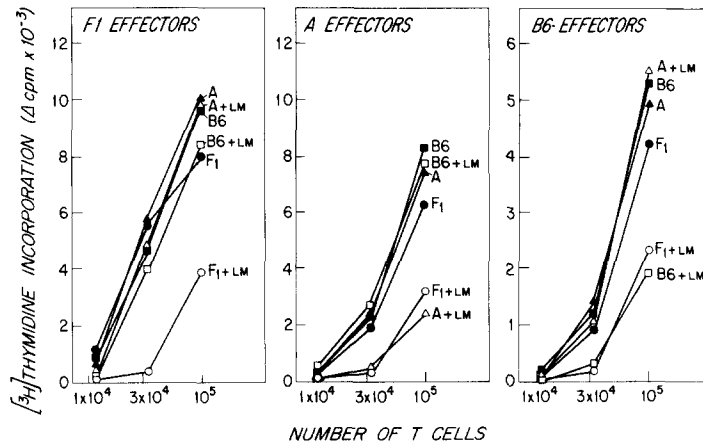


FIG. 8. *L. monocytogenes*-immune T cells from B6AF₁ mice were fractionated on macrophage monolayers derived from B6AF₁ (F₁), C57BL/6 (B6), and A/St (A) mice. Macrophage monolayers which received *L. monocytogenes* pretreatment are designated: + LM. T cells nonadherent to the macrophage monolayers were then tested for function on B6AF₁-, A/St-, and C57BL/6-derived effector macrophages (F₁-EFFECTORS, A-EFFECTORS, B6-EFFECTORS). Results of the mitogenic protein assay are shown.

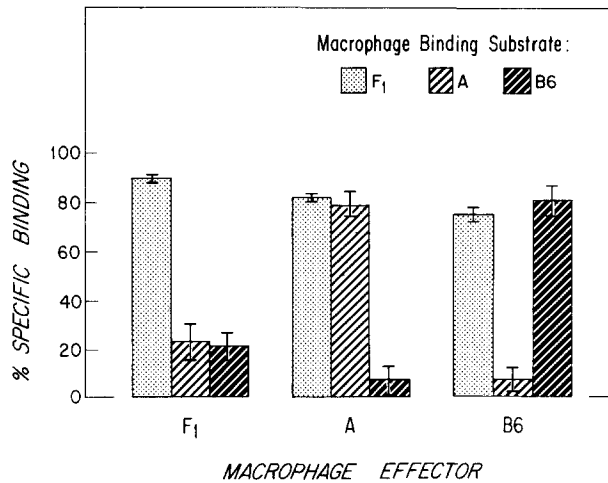


FIG. 9. The results of two separate experiments performed as described in Fig. 8 are given as the arithmetic mean of the percentage of specific binding plus or minus range. The percentage of specific binding in both experiments was calculated (as described in Materials and Methods) with data from the mitogenic protein assay at 3.3×10^4 fractionated T cells.

ment. Both *L. monocytogenes*-pulsed and control macrophages from B6AF₁ mice were then incubated for 1 h at 37°C with a 1:50 dilution of B10.D2 anti-B10.A serum. After removal of the antiserum by washing, *L. monocytogenes*-immune T cells from B6AF₁ mice were added, and the binding reaction performed as usual. The activity of fractionated T cells was then measured on A/St and C57BL/6 effector macrophages. The data derived from the mitogenic protein assay are given as the percentage of specific binding calculated from values for two T-cell densities. As can be clearly seen, the binding of the clone of B6AF₁ T cells reactive with A/St effector macrophage

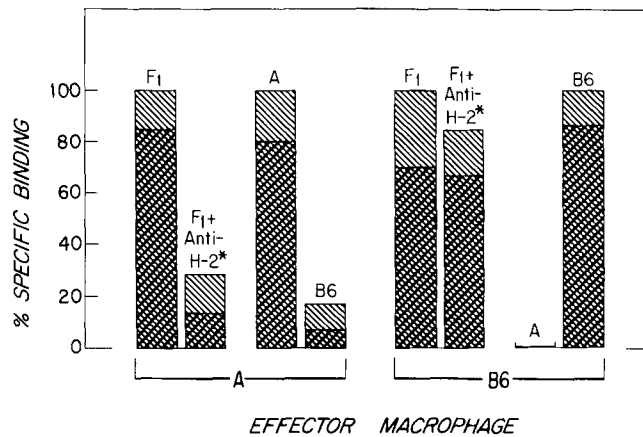


FIG. 10. *L. monocytogenes*-immune T cells from B6AF₁ mice were fractionated on various binding macrophages as indicated above the bars (F₁, B6AF₁; F₁ + Anti-H-2 [Anti-H-2: B10.D2 anti-B10.A]; A, A/St; B6, C57BL/6.) and then tested for function on effector macrophages from A/St (A) or C57BL/6 (B6) mice. The binding reaction was performed exactly as described previously (e.g., Fig. 1) except that B6AF₁-binding macrophages, after pretreatment with *L. monocytogenes* (2×10^7 *L. monocytogenes* per ml, 1 h, 37°C), were incubated for an additional hour in the presence of a 1:50 dilution of B10.D2-anti-B10.A serum before washing and addition of *L. monocytogenes*-immune T cells. The percentage of specific binding was calculated (as described in Materials and Methods) with data from the mitogenic protein assay at two T-cell densities (3.3×10^4 and 1×10^5) indicated by the different shadings of the bar graph. (Hatched bar, 3.3×10^4 ; cross-hatched bar, 10^5).

is markedly inhibited, whereas the binding of the C57BL/6-reactive clone is unaffected.

Similar experiments were performed with antibodies directed against Ia antigens (A.TH-anti-A.TL). *L. monocytogenes*-pulsed binding macrophages from A/St mice were incubated (1 h, 37°C) with a 1:10 dilution of either normal mouse serum (NMS) or A.TH-anti-A.TL serum and then washed before the initiation of the binding reaction with *L. monocytogenes*-immune A/St T cells. A typical experiment showed that NMS-treated macrophages supported 54% specific binding, whereas those pretreated with anti-Ia antibodies failed to show significant specific binding (3%). Additionally (as in Fig. 10), anti-Ia (A.TH-anti-A.TL) could selectively inhibit the binding of the A/St-reactive clone of B6AF₁ T cells to B6AF₁ binding macrophages (data not shown).

Discussion

A system has been developed to quantitate the antigen-specific binding of T cells to macrophages. The concordant studies of Lipsky and Rosenthal (20) and Lipscomb, et al. (21), with systems in which the binding of guinea pig T cells to macrophages was quantitated on the basis of cell number, are confirmed and extended here by utilizing a bacterial antigen and mouse lymphoid cells. Our system has two major advantages. First, the binding reaction times are very short; second, and most significant, the binding can be monitored by the relative depletion or enhancement of specific T-cell function. The key to the short reaction time is the light centrifugation step (Table I). The functional monitoring was greatly facilitated by the previous development of sensitive systems to measure T-cell-macrophage effects not requiring the assay of T-cell proliferation (11, 12).

The physical interactions described here appear to be assaying the antigen recognition stage of macrophage-T-cell interactions. This system is a suitable model for antigen recognition for a number of reasons. First, the antigen-specific physical interaction between specific T cells and macrophages can be evidenced both by the decrease in specific T cells in the macrophage nonadherent cells, as well as by the relative increase in specific cells in the macrophage-bound fraction, as evidenced by the stimulation of the binding macrophage to which the T cells are bound and by the enhanced activity of recovered T cells. Second, both depletion and uptake can be measured on a cell-number basis, as well as by quantitation of relative T-cell function. Third, the interaction occurs rapidly and to a significant degree at 4°C (albeit substantially less than at 37°C). Furthermore, the functional depletion could not be explained by induction of a suppressive effect in the recovered T cells because mixtures of fractionated and unfractionated T-cell populations yielded an activity equivalent to the simple addition of the two populations when measured separately (data not shown). These findings provide evidence that true binding occurs. Although measurements of cell number and function give assuringly similar results, the quantitation of binding by functional depletion yields the most definitive evidence when attempts are made to correlate binding ability with a given T-cell activity.

Previous studies from our laboratory indicated that Ir genes modulated the *L. monocytogenes*-T-cell-macrophage function described herein. Careful mapping using congenic mouse strains pointed to the I-A as the major locus controlling the interactions (11, 12). Our present results support an involvement of H-2 in the specific binding reaction. First, in keeping with the genetic restrictions observed when function was measured, antigen-specific binding ability mapped to the left-hand region of the H-2 gene complex (K through I-E) (Fig. 6). Mapping studies with congenic strains are in progress. Second, at least two distinct populations of B6AF₁ T cells with binding avidity for antigen presented on parental macrophages could be identified (Figs. 8 and 9). Third, the binding of a given parental-reactive F₁ T-cell clone could be specifically inhibited by pretreatment of the antigen-pulsed B6AF₁-binding macrophages with anti-H-2 (K through I-E) antibodies reactive with the appropriate parental haplotype (Fig. 10). Additionally, specific binding was blocked with antibodies directed against the Ia antigens of the binding macrophages. These results provide direct evidence that anti-Ia antibodies inhibit T-cell function at the macrophage level. The involvement of other cell-surface molecules has not been critically addressed.

The ability of anti-H-2 antibodies to inhibit a number of antigen-specific T-cell functions has been well documented (22). Given the known requirements for Ia antigen-bearing macrophages in these functional systems and the ability of anti-H-2 and, specifically, of anti-Ia antibodies to inhibit specific T-cell-macrophage binding (Results; 23, 24), it is tempting to speculate that all of the inhibitory influences of anti-Ia reagents are mediated through a common mechanism: the prevention of the physical liaison between T cells and macrophages.

The results presented herein, together with the studies with guinea pig models, imply that antigen-specific T-cell-macrophage binding is the first, and thus determining, event in Ir-gene immune reactivity. It should be emphasized, however, that the binding event itself may well be dictated by even earlier activation events. This possibility is worthy of inquiry because of the observations that specific T-cell-

macrophage binding is temperature dependent (Table I) and that antigen-dependent binding appears to be preceded by a transient antigen-independent interaction (Figs. 2 and 3). Additionally, recent studies on the separation of recognitive and proliferative phases of T-cell activation by Greenberg and Bluestein (25) support the concept that multiple events culminate in Ir-gene-controlled T-cell activation as measured by antigen-dependent proliferation. These authors have suggested that an antigen-dependent, macrophage-independent, and Ir-gene-independent activation precedes a macrophage-dependent and Ir-gene-dependent second signal (suggested to be T-cell-macrophage binding) which leads to proliferation of antigen-specific T cells. Our studies lend direct evidence for this thesis; furthermore, our observations on the enhanced production of a protein which causes a nonspecific T-cell proliferation (i.e., mitogenic protein) may well represent this second signal.

The question remains: What does the temperature dependency of a specific T-cell-macrophage binding mean in terms of the possible antigen-T-cell-macrophage communication which leads to the H-2-restricted binding event? On one hand, the temperature dependency may reflect the redistribution of T-cell and macrophage recognition molecules (e.g., T-cell-antigen receptors, antigen, and/or I region products) to sites of cell-cell contact which would increase the binding avidity by favoring multivalent cooperative interactions (26). Alternatively, one may envision distinct, sequential, and interdependent events that proceed optimally at temperatures $>4^{\circ}\text{C}$. These putative events may include the postulated association of antigens with Ia molecules (15, 27), enzymatic alterations of antigen or Ia molecules (8), or an event involving the exocytosis or display of antigen determinants (Ia and/or antigen) previously sequestered by the macrophage. One of many such models may be that the initial antigen-independent T-cell-macrophage interaction programs the cells for subsequent antigen-dependent binding which, in turn, causes T-cell activation. For example, the initial T-cell-macrophage interaction may cause the appropriate antigenic determinant and/or Ia molecule to be displayed only at the T-cell-macrophage junction. The possible inaccessibility of an antigen-containing recognition unit at the cell-cell interface would supply an explanation for the lack of inhibition by antibody directed against intact antigen.

The system discussed here should permit a systematic and definitive analysis of the Ir gene-directed clonality of the T-cell repertoire. Our demonstration of two clones of *L. monocytogenes*-specific B6AF₁ T cells directed against parental macrophages (Fig. 9) is in keeping with other reports on the clonality of B6AF₁ T cells (8, 17-19). Unlike the studies presented here, however, previous studies have usually employed positive- and negative-selection procedures which required the appropriate T cells to clonally expand or be eliminated upon proliferation in the presence of bromodeoxyuridine and light. Thus, our studies represent a significant extension of these findings by physically separating the clones by using relatively short periods of time (1 h) and by directly addressing the role of H-2 gene products in the binding reaction. It remains to be determined, however, if antigen-specific H-2-directed responses show binding restrictions at the precursor T-cell level. Furthermore, the identification of a third clone of *L. monocytogenes*-specific B6AF₁ T cells reactive specifically with the F₁ macrophage and not with either parental macrophage has been accomplished (K. Ziegler and E. R. Unanue, Manuscript in preparation.). This finding, predicted on the basis of studies on gene complementation (28), B6AF₁-specific mixed lymphocyte

reactivity (29), and biochemical characterization of murine Ia antigens (30) raises interesting questions as to the presence of B6AF₁-specific clones possibly determined by a two-gene control (I-A and I-E/I-C) of Ia antigen expression (30). As has been previously argued for H-2K- and H-2D-restricted cytotoxic responses to virus-infected cells (31), the multiple clonal response to *L. monocytogenes* (as demonstrated in this paper) serves to illustrate the survival advantage for the polymorphism of Ia antigens as a mechanism by which multiple clones are generated such that the likelihood of a nonresponder situation in heterozygote animals is decreased. Thus, the enumeration of *L. monocytogenes*-reactive clones in outbred mice against homozygote macrophages, together with the determination of the possible existence of separate clones of T cells reactive with individual Ia subregions, will be of interest.

In summary, our results, together with similar studies with guinea pig models, have led to the conclusion that Ir gene products play a direct role in cell-cell contact rather than only a triggering role in the expression of macrophage-dependent T-cell function. It should be emphasized, however, that these findings do not exclude the possibility that Ir gene products are indeed the triggering and/or recognitive structures in developmental and/or inductive processes which generate the T-cell repertoire. Such structures (e.g., Ir gene products), known to elicit vigorous mixed lymphocyte reactivity (32), may well be the recognitive and triggering molecules that drive the generation of the diversity in the thymus as proposed by Jerne (33).

Summary

A system was developed to study the binding of *Listeria monocytogenes*-specific T cells to *L. monocytogenes*-pulsed macrophages as an analogue of the initial phase of T-cell activation: antigen recognition. Specific binding, demonstrable after a brief (1 h) contact, was quantitated by the depletion of *L. monocytogenes*-specific T-cell activity in the cells nonadherent to *L. monocytogenes*-pulsed macrophage monolayers. *L. monocytogenes*-specific T-cell function was measured by its ability to activate *L. monocytogenes*-pulsed macrophages, both to secrete a protein mitogenic for thymocytes and to effect nonspecific tumoricidal activity. These manifestations of T-cell function are known to be regulated by products of I region of the H-2 gene complex. Studies designed to determine the role of H-2 gene products in specific T-cell-macrophage binding have revealed the following. T cells bind specifically to syngeneic macrophages and poorly to allogeneic macrophages. The binding ability appears to map to the K end of the H-2 gene complex (K through I-E). At least two distinct populations of B6AF₁ T cells with binding avidity for *L. monocytogenes* presented on parental macrophages can be identified. Finally, the binding of a given parental-reactive B6AF₁ T-cell clone can be specifically inhibited by pretreatment of the antigen-pulsed B6AF₁ binding macrophage with anti-H-2 (anti-Ia) antibodies reactive with the appropriate parental haplotype. These results strongly suggest that H-2 gene products play a direct role in mediating the specific binding of T cells to macrophages and imply that the antigen-dependent physical interaction between T cells and macrophages is the initial, and determining, event in some forms of H-2 gene control of immune reactivity.

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