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The mechanism of macrophage-antigen handling was studied using a system that involves the quantitation of the antigen-specific binding of *Listeria monocytogenes*-immune T cells to macrophages. Specific T cells did not bind to native antigen. Because the specific binding of T cells to macrophages could be measured during a short (5- to 15-min) interaction, it was possible to follow the temporal development of a T cell-binding substrate with increasing time of antigen-macrophage interaction. In contrast to the rapid (5-min) uptake of *Listeria* by macrophages, the development of T cell-binding ability required a 30- to 60-min period of antigen-macrophage interaction. During this processing period, Listeria organisms bound to the macrophage surface were ingested and partially catabolized. Unlike antigen uptake, antigen processing was a temperature-dependent and energy-requiring event. Although macrophages treated with paraformaldehyde before antigen processing did not develop T cell-binding activity, macrophages treated with paraformaldehyde after a 60-min antigen-processing period retained T cell-binding ability. The kinetics of antigen catabolism correlated with antigen processing, and inhibition of antigen catabolism was associated with a corresponding inhibition of antigen processing for T cell binding. Anti-la antibodies had no effect on *Listeria* uptake or catabolism. These results supply direct evidence for a macrophage-antigen processing event relevant to T cell recognition of antigen.

Macrophages are intimately involved in the antigen-specific activation of Ly1+2-3- T lymphocytes (reviewed in 1-3). The initial event in T cell activation is thought to be the recognition of the immunogen and I region gene products present on the macrophage cell surface (3, 4). In the present study, we attempt to correlate the uptake, ingestion, and catabolism of antigen by the macrophages to their ability to present immunogenic molecules to T cells. To this effect, we have used the binding of specific T cells to macrophages as a functional assay of macrophage-associated antigens. It is known that the interactions between T cells and macrophages bearing antigen may be analyzed directly by quantitating the antigen-specific physical interactions taking place between both cells (5-13). This quantitation of macrophage-associated antigenicity within a short temporal framework is a unique way to study the mechanisms of macrophage handling of antigen in the context of I region gene effects. Previous studies have approached this problem by using radioactively labeled antigen molecules to follow their fate in phagocytic cells, and long-term functional assays for the immunogenicity of macrophage-associated antigen (14-16). The phagosome-lysosome pathway of antigen uptake and degradation and the potent immunogenicity of macrophage-associated antigen have been well illustrated. However, because of the dynamic nature of macrophage-antigen handling events and the length of time required to functionally assess antigenicity, precise cause-and-effect relationships between antigen-handling events and immune recognition could not be determined with certainty.

We have employed as antigen the intracellular pathogenic bacteria *Listeria monocytogenes*. The activation of *Listeria* immune T cells requires macrophages in a process regulated by the I region of the H-2 (11, 12, 17). Moreover, *Listeria* can be traced in the macrophage and its uptake, ingestion, and catabolism monitored reasonably well. We now define a macrophage processing event required for the T cell recognition of *Listeria* antigen.

### MATERIALS AND METHODS

**Mice.** A/St mice were purchased from West Seneca Laboratories, Buffalo, NY. Male or female mice at 8 to 12 wk of age were employed. Heat-killed *Listeria monocytogenes*. The preparation of *Listeria monocytogenes* was previously described (17). Live bacteria were used to immunize mice, and heat-killed organisms served as antigen for use in vitro.

Heat-killed *Listeria monocytogenes* were labeled with *125* by the chloramine-T method (18). Briefly, 10^6 washed bacteria suspended in 130 μl of phosphate-buffered saline (PBS) containing 1 mCi of carrier-free Na*251 (New England Nuclear, Boston, MA) and 50 μg of chloramine-T (Eastman Kodak, Rochester, NY) were incubated for 10 min at 4°C. Sodium metabisulfite (Sigma Chemical Company, St. Louis, MO) (20 μl of a 5 mg/ml solution) was added to terminate the reaction, and then the labeled bacteria were washed 5 times in PBS to remove free *125*. Bacteria thus treated showed 1 to 2 counts per minute (cpm) per bacterium. More than 99% of the radioactivity was precipitable in 10% trichloroacetic acid (TCA). *Listeria monocytogenes* labeled with *125* (3*10^8*-Listeria) were stored at 4°C in PBS for use over several weeks and were washed immediately before use.

In 1 experiment, *Listeria monocytogenes* were attached to culture dishes using poly-lysine. Tissue culture wells (16 mm diameter) were treated (1 hr, 20°C) with an aqueous solution of poly-lysine (1 mg/ml) and then washed. Heat-killed *Listeria* in PBS (0.5 ml of 10^6* bacteria/ml) was added, the plate was centrifuged (800 X G, 5 min), and then incubated for 1 hr at 20°C. After washing with PBS, a confluent lawn of bacteria remained firmly attached to the dish.

**Uptake and ingestion of *Listeria* by macrophages.** Labeled *Listeria monocytogenes* were added to 3 X 10^6* peritoneal exudate cells (PEC) planted in 16-mm diameter tissue culture wells (Falcon No. 3008). The plates were centrifuged (800 X G, 5 min) and then incubated at 37°C for 6-18 hr before fixation with 1% paraformaldehyde. The *Listeria* monocyto-
ocytogenes organisms on the surface of the macrophages remained reactive with anti-Listeria antibody and could be visualized by indirect immunofluorescence. Inverted coverslips were incubated (30 min, 4°C) on 20 μl of a 1:10 dilution of rabbit anti-Listeria serum, washed, and then treated with fluorescein-conjugated F(ab')2 goat anti-rabbit globulin (20 μl of 50 μg/ml). Catabolism of 125I-Listeria by macrophages. Macrophage monolayers were cultured with 125I-Listeria to allow uptake (as described above) and then washed thoroughly. After incubation for various periods of time, the supernatant was removed, Listeria-immune T cells were added to the macrophages. The solubilized macrophages were recovered from the dish with the aid of a rubber policeman. Both the macrophage and supernatant fractions were incubated with an equal volume of 20% TCA, and the precipitate was separated by centrifugation (1000 g, 30 min, 4°C). Radioactivity was measured in the precipitate and in the soluble supernatant.

**Results**

The binding assay. The antigen specificity and macrophage dependence of antigen recognition by Listeria-immune T cells is illustrated by the experiment of Table I. Listeria monocytogenes-immune T cells were added to tissue culture dishes containing the various binding substrates. Binding was monitored by testing the nonadherent T cells in the macrophage cytotoxicity assay. Although complete depletion of Listeria-specific T cell activity was observed with macrophages incubated with Listeria, no binding was observed with macrophages incubated with the antigenically unrelated bacteria, Salmonella typhi. Furthermore, Listeria-immune T cells did not bind to Listeria monocytogenes attached to the dish in the absence of macrophages. The inability of immobilized Listeria monocytogenes to support binding was also observed when the mitogenic protein and proliferation assays were used to assess T cell activity (data not shown). Clearly, both antigen and macrophages are required to generate a substrate to which T cells will bind.

Kinetics of antigen handling. Several aspects of the handling of Listeria by macrophages were studied. Macrophages from Listeria monocytogenes-immune mice containing a high proportion of la-positive cells were used (21). Many such macrophages have been shown to bind Listeria-immune T cells much better than macrophages from peptone-induced PEC (unpublished observations). The uptake, ingestion, and catabolism of Listeria by macrophages as function of time are shown in Figure 1.

Substantial uptake of Listeria occurred within 5 to 10 min by using a centrifugation step (800 × g, 5 min) to initiate the interactions of bacteria with macrophage monolayers (Fig. 1a). Routinely, 15 to 30% of the added 125I-Listeria became macrophage associated during this time period. Rapid (5-minute) uptake occurred equally well in the presence of both sodium azide (10−2 M) and 2-deoxyglucose (10−3 M), and also at 4°C (data not shown).

After the rapid binding (5 min) of Listeria to the macrophage cell surface, ingestion was evidenced by the progressive decrease in the number of macrophage surface-associated bacteria, reaching a 50% reduction by about 10 min (Fig. 1b).

In Figure 1c, macrophages were exposed (30 min 37°C) to 125I-Listeria to allow for uptake and ingestion, then washed thoroughly; the radioactivity in the macrophage and the super-

<table>
<thead>
<tr>
<th>Binding Substrate</th>
<th>Activity of Nonadherent T Cells: Macrophage-Mediated Percent (± SEM)</th>
<th>% Specific Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>39.5 ± 3.9</td>
<td>11</td>
</tr>
<tr>
<td>Macrophages and Salmonella</td>
<td>43.9 ± 2.1</td>
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</tr>
<tr>
<td>Macrophages and Listeria</td>
<td>5.4 ± 1.6</td>
<td>100</td>
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<tr>
<td>Culture dish</td>
<td>42.7 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Salmonella</td>
<td>47.3 ± 0.6</td>
<td>11</td>
</tr>
<tr>
<td>P-815 mastocytoma</td>
<td>50.3 ± 2.7</td>
<td>18</td>
</tr>
</tbody>
</table>

* Macrophage monolayers (1.5 × 106 PEC per well) were incubated (1 hr, 37°C) with heat-killed Salmonella typhi or Listeria monocytogenes (0.3 μl of 10−6 bacteria per ml). Some culture dishes contained bacteria attached to the culture surface. Listeria-immune T cells (5 × 106 T cells per well) were added to the dishes containing the indicated binding substrate; the plates were centrifuged (50 × g, 5 min, 20°C) and then incubated at 37°C for 1 hr. The T cells nonadherent to the binding substrates were recovered and tested for specific activity in the macrophage cytotoxicity assay. The activity of 105 cells per well is given as the mean percent specific cytotoxicity ± SEM of duplicate binding reactions.
The uptake of 125I-labeled Listeria monocytogenes by macrophages was shown as a function of time. 125I-Listeria (0.5 ml per well, 10^5 cpm per well) was added to tissue culture wells containing macrophage monolayers formed with 10^6 PEC. The plates were centrifuged (800 x G, 5 min), incubated for the indicated periods of time at 37°C, and then washed to remove unbound bacteria. The macrophage-associated radioactivity recovered after treatment with 1% Triton X-100 is shown (closed symbols). Open symbol represents 125I-Listeria associated with culture well in the absence of macrophages. a. The ingestion of Listeria monocytogenes by macrophages is shown as a function of time. Time zero represents macrophages exposed to Listeria for a 5-min centrifugation period (as in Fig. 1a), washed, and fixed immediately. c. The catabolism of 125I-Listeria macrophages was followed with time. Macrophage monolayers (10^6 PEC per well) were exposed (30 min) to 125I-Listeria to allow uptake and ingestion as in Figures 1a and 1b and then incubated for various periods of time at 37°C. Trichloroacetic acid- (10%) soluble (open symbols) and -precipitable (closed symbols) radioactivity was followed in the macrophage (triangles) and in the supernatant (circles).

\[\text{UPTAKE}\]

\[\text{INGESTION}\]

\[\text{CATABOLISM}\]

Figure 1. a. The uptake of 125I-labeled Listeria monocytogenes by macrophages is shown as a function of time. 125I-Listeria (0.5 ml per well, 10^5 cpm per well) was added to tissue culture wells containing macrophage monolayers formed with 10^6 PEC. The plates were centrifuged (800 x G, 5 min), incubated for the indicated periods of time at 37°C, and then washed to remove unbound bacteria. The macrophage-associated radioactivity recovered after treatment with 1% Triton X-100 is shown (closed symbols). Open symbol represents 125I-Listeria associated with culture well in the absence of macrophages. b. The ingestion of Listeria monocytogenes by macrophages was monitored visually by indirect immunofluorescence with an anti-Listeria antibody. Bacteria on the macrophage cell surface but not those that are ingested are reactive with the antibody. Each point represents the number of surface bacteria per macrophage (mean ± SEM). Two different experiments are indicated by the different symbols. Time zero represents macrophages exposed to Listeria for a 5-min centrifugation period (as in Fig. 1a), washed, and fixed immediately. c. The catabolism of 125I-Listeria macrophages was followed with time. Macrophage monolayers (10^6 PEC per well) were exposed (30 min) to 125I-Listeria to allow uptake and ingestion as in Figures 1a and 1b and then incubated for various periods of time at 37°C. Trichloroacetic acid- (10%) soluble (open symbols) and -precipitable (closed symbols) radioactivity was followed in the macrophage (triangles) and in the supernatant (circles).

Macrophages tested for T cell binding immediately after antigen uptake showed little or no specific binding, whereas the other was incubated for 60 min at 37°C before addition of T cells. Thus, the antigen-uptake phase (5 min) and the T cell-macrophage binding reaction (15 min) were held constant. Macrophages tested for T cell binding immediately after antigen uptake showed little or no specific binding, whereas those incubated for 60 min at 37°C before the T cell-macrophage reaction showed substantial binding of T cells. Clearly, a period of Listeria-macrophage interaction after simple antigen uptake is required for the generation of a T cell binding substrate. Increasing the amount of Listeria 10-fold did not overcome this requirement. This operationally defined time period of Listeria-macrophage interaction will be termed processing.

To more carefully analyze the temporal requirements for processing, several experiments were performed by varying the time of Listeria-macrophage interaction. In the experiment shown in Figure 3, both the time period macrophages were exposed to Listeria before addition of T cells (processing time) and the times of T cell-macrophage interaction (binding time) were varied. Little or no specific binding was observed when the combined binding time and processing time was less than 30 min. It appeared that the presence of T cells for various lengths of time had no substantial influence on the temporal progression in the development of T cell-binding substrate, since the magnitude of specific binding was directly related to.

\[\text{Figure 2.} \quad \text{Macrophage monolayers (MAC) were exposed (5 min, 800 x G, 20°C) to heat-killed Listeria monocytogenes (LM) (0.3 ml of 10^5 or 10^6 bacteria per ml), washed, and then incubated for 0 or 60 min at 37°C before addition of T cells (5 x 10^5 per well in 0.5 ml). T cell-macrophage interaction initiated by a brief centrifugation (50 x G, 5 min) was conducted for a total of 15 min at 37°C. Percent specific binding was monitored by the readout systems as indicated and percent specific binding calculated as described in Materials and Methods. The mean ± SEM for duplicate binding reactions is shown. Control values for the activity of T cells exposed to macrophage monolayers in the absence of Listeria were 12,401 ± 410 dcpm for mitogen protein and 7,853 ± cpm for proliferation.}\]
the total time of handling of the bacterium, i.e., the binding time plus processing time.

The pooled results of 9 experiments in which specific binding was measured as a function of the total time of Listeria-macrophage interaction is shown in Figure 4. The time indicated represents the period beginning with the addition of Listeria to the macrophage and ending with the removal of T cells from the macrophage monolayers. The T cell-binding ability developed after a lag period of about 20 min to maximal levels by 60 min. One-half maximal binding was observed at 45 min.

In a control experiment, macrophages were incubated with Listeria for 60 min and were then given Listeria for a 2nd time immediately before addition of T cells. Specific binding was not inhibited (82% specific binding after 1 hr of Listeria vs 86% binding after the 2 exposures). We considered that the Listeria bound to the macrophage cell surface did not interfere with T cell binding.

Processing requires active macrophage metabolism. If antigen handling was carried out in the presence of sodium azide (10^-2 M) and 2-deoxyglucose (10^-1 M), the generation of a T cell-binding substrate was markedly inhibited (80% inhibition) (Table II). Likewise, incubation of macrophages with bound Listeria at 4°C markedly reduced the binding of T cells (Table II). Inhibitors of protein synthesis did not affect the binding of T cells to macrophages (Table II).

The need for metabolically active macrophages during the antigen-processing period was also apparent when testing the binding ability of paraformaldehyde-treated macrophages (Fig. 5). T cells did not bind to macrophages treated (5 min, 20°C) with 1% paraformaldehyde immediately after antigen uptake. However, substantial (approximately 60% of control) T cell binding was observed to macrophages allowed to process Listeria for 50 min at 37°C before fixation.

Role of la in antigen handling. As indicated in previous studies, the exposure (30 to 60 min, 37°C) of macrophages to antibodies directed against I-region gene products resulted in inhibition of T cell-macrophage binding (11). With an A.TH-anti-A.TL serum (anti-la') at 1:10 dilution, 70 to 90% inhibition of specific binding was routinely observed; a monoclonal anti-

### Table II

<table>
<thead>
<tr>
<th>Binding Substrate</th>
<th>Treatment</th>
<th>Mitogenic Protein Activity of Macrophage Nonadher-ent T Cells, 3TClm ± SEM</th>
<th>% Specific Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
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<tr>
<td>Macrophages</td>
<td>None</td>
<td>7.629 ± 0.87</td>
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<td>Macrophages and</td>
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<td>Listeria</td>
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<tr>
<td>Macrophages</td>
<td>8.670 ± 0.12</td>
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<td>4.498 ± 0.23</td>
<td>38</td>
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<tr>
<td>Listeria</td>
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<tr>
<td>Macrophages</td>
<td>7.086 ± 0.50</td>
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<td>Listeria</td>
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<td></td>
<td></td>
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<tr>
<td>Macrophages</td>
<td>7.501 ± 0.10</td>
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<td>Listeria</td>
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<td></td>
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<tr>
<td>Macrophages</td>
<td>7.930 ± 0.37</td>
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<td>4.025 ± 0.47</td>
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<tr>
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<td></td>
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</tr>
<tr>
<td>Macrophages</td>
<td>7.490 ± 0.30</td>
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<tr>
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<tr>
<td>Listeria</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>8.351 ± 0.32</td>
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<td>Macrophages and</td>
<td>7.247 ± 0.47</td>
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<tr>
<td>Listeria</td>
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<tr>
<td>Macrophages</td>
<td>14.139 ± 0.80</td>
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<tr>
<td>Macrophages and</td>
<td>2.654 ± 0.34</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>Listeria</td>
<td>4°C</td>
<td>13.312 ± 0.47</td>
<td>6</td>
</tr>
</tbody>
</table>

*Macrophage monolayers were prepared as binding substrates by using PEC from Listeria-immune mice (1.5 x 10^7 PEC per well). In Experiment 1, monolayers were incubated (30 min, 37°C) with the inhibitors at the indicated concentrations in a total volume of 0.5 ml. Heat-killed Listeria monocytogenes (50 μl per well of 10^7 bacteria per ml) were added as indicated; the plates were centrifuged (800 x G, 5 min) and then incubated for 30 min at 37°C in the continued presence of the inhibitor. After Listeria-macrophage interaction, the macrophages were washed to remove the inhibitors and unbound bacteria. Listeria-immune T cells were added (5 x 10^5 per well in 0.5 ml), the plate centrifuged (800 x G, 5 min), and then incubated at 37°C for 20 min. The T cells nonadherent to the macrophage monolayers were recovered, washed, counted, and then tested for activity in the mitogenic protein assay. The activity of 10^5 cells per well is given as cpmp (mean ± SEM) of duplicate binding reactions. Percent specific binding was calculated by using the activity of T cells nonadherent to macrophages that received no treatment with Listeria. The protein synthetic capacity of macrophages measured in parallel by using ^3H-leucine incorporation was inhibited over the time period of Listeria-macrophage and macrophage-T cell interaction. For example, with 10 μg per ml cycloheximide, 80% inhibition of ^3H-leucine incorporation was observed. In Experiment 2, the macrophage monolayers were incubated with Listeria monocytogenes to allow uptake by macrophages and then incubated for 60 min at either 37°C or 4°C. T cells were added, and the binding reaction was carried out for 15 min at 37°C.

IAA' reagent (neat) showed 30 to 60% inhibition. Inhibition of T cell-macrophage binding with these reagents was observed even when macrophages were exposed to the antibodies before addition of antigen. For example, when macrophages were exposed (30 min, 37°C) to the anti-IAA' reagent, either before or after a 30-min period of Listeria-macrophage interaction, 30 ± 7% or 35 ± 5% inhibition (mean ± SEM, 3 experiments) of specific binding, respectively, was obtained. It was of interest, therefore, to address the possible role of macrophage la molecules in the handling of antigen by macrophages. In the experiment shown in Table III, macrophages exposed to anti-la antibodies were tested for their ability to bind and catabolize ^125I-labeled Listeria. No effect on the uptake or catabolism or antigen was observed under the conditions known to inhibit specific T cell-macrophage binding.
The major thrust of this study was to analyze the kinetics of antigen uptake, ingestion, and catabolism by macrophages and to relate such events to the ability of macrophages to serve as a binding substrate for antigen-specific T lymphocytes. The system that we chose for analysis of antigen-handling events clearly involves the recognition of Listeria antigen by T cells in the context of I-region products of the macrophage. Because the specific binding of T cells to macrophages could be measured during a short (5- to 15-min) incubation period, it was possible to follow the temporal development of a T cell-binding substrate with increasing time of antigen-macrophage interactions (summarized in Fig. 6). We found that T cell binding to macrophages developed after a 30- to 60-min period of antigen handling. These kinetics were in marked contrast to the rapid uptake and ingestion of bacteria by the macrophages, indicating that antigen uptake alone—a surface event—was insufficient to generate a macrophage-associated immunogen for T cell binding. These results formed the basis for the operational definition of macrophage post-antigen-uptake events required for antigen recognition by T cells as “antigen processing.”

Antigen processing events may also be dissociated from antigen uptake on the basis of temperature and energy dependence. Although simple antigen uptake by macrophages can occur at 4°C or in the presence of inhibitors of oxidative and glycolytic metabolism, azide, and 2-deoxyglucose, little or no T cell-binding ability develops under these conditions (Table II, Fig. 6). These results are in keeping with previous studies (22, 23) which suggested that the accumulation of immunogenically relevant antigen by macrophages proceeds by membrane binding and then subsequent metabolic-dependent sequestration of bound antigen. Our findings that the ingestion of bacteria by the macrophage precedes the development of T cell-binding ability is in keeping with this pathway of antigen handling.

The requirement for active macrophage events during antigen processing was also apparent when the binding ability of paraformaldehyde-fixed macrophages was studied (Fig. 5). We conclude that once antigen processing by macrophages is

<table>
<thead>
<tr>
<th>Treatment of Macrophages</th>
<th>% Antigen Uptake (5 Min)</th>
<th>% Antigen Catabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>30</td>
<td>8.3</td>
</tr>
<tr>
<td>Anti-I-A* (undiluted), 30 min</td>
<td>28</td>
<td>7.4</td>
</tr>
<tr>
<td>Anti-I-A* (1:10), 30 min</td>
<td>33</td>
<td>6.5</td>
</tr>
</tbody>
</table>

* Macrophage monolayers were prepared from the PEC (10^6 per well) of Listeria monocytogenes-immune mice. Macrophages in this experiment were 76% IA-positive as judged by indirect immunofluorescence. Macrophages were incubated (30 min, 37°C) with either a monoclonal anti-I-A* antibody preparation or an A.TH anti-A.TL serum (anti-I-A*). ^125I-Listeria (10^6 cpm per well) was added; the plates were centrifuged (800 X G, 5 min), and then the ^125I-Listeria not bound to macrophages removed by washing. The macrophages bound with ^125I-Listeria were then incubated for various periods of time at 37°C, and the TCA-soluble (10%) radioactivity released into the supernatant was assessed. Antibodies were present throughout the 37°C incubation period.

Evidence for the ingestion of bacteria by the macrophages was provided in Table III. In this experiment we chose for analysis of antigen-handling events that antigen uptake alone was insufficient to generate a macrophage-associated immunogen for T cell binding. These results formed the basis for the operational definition of macrophage post-antigen-uptake events required for antigen recognition by T cells as “antigen processing.”

**DISCUSSION**

The major thrust of this study was to analyze the kinetics of antigen uptake, ingestion, and catabolism by macrophages and to relate such events to the ability of macrophages to serve as a binding substrate for antigen-specific T lymphocytes. The system that we chose for analysis of antigen-handling events clearly involves the recognition of Listeria antigen by T cells in the context of I-region products of the macrophage. Because the specific binding of T cells to macrophages could be measured during a short (5- to 15-min) incubation period, it was possible to follow the temporal development of a T cell-binding substrate with increasing time of antigen-macrophage interactions (summarized in Fig. 6). We found that T cell binding to macrophages developed after a 30- to 60-min period of antigen handling. These kinetics were in marked contrast to the rapid uptake and ingestion of Listeria by the macrophages, indicating that antigen uptake alone—a surface event—was insufficient to generate a macrophage-associated immunogen for T cell binding. These results formed the basis for the operational definition of macrophage post-antigen-uptake events required for antigen recognition by T cells as “antigen processing.”
complete, then the macrophage cell surface serves to display antigen that T cells recognize. Thus, a mechanism of T cell-antigen recognition involving an active T cell-induced display of antigen previously sequestered by the macrophage seems unlikely. The abilities of aldehyde-fixed tumor cells to serve as targets for T cell-mediated cytotoxic attack (24) and aldehyde-fixed TNP-modified macrophages to stimulate TNP-specific T cell proliferation (25) have been noted previously.

The possible role of antigen catabolism in the macrophage-handling events relevant to T cell-antigen recognition is suggested by several pieces of evidence. First, T cells did not bind to intact antigen (Table I). Second, the kinetics of antigen catabolism correlated most closely with the kinetics of development of a T cell-binding substrate (Fig. 5). Third, both the appearance of T cell-binding ability and antigen catabolism were inhibited by low temperature, metabolic energy inhibition, and aldehyde fixation. Also, in results to be published, ammonium chloride, a well-studied inhibitor of protein degradation in cultured cells, resulted in a substantial inhibition of antigen catabolism without effect on antigen uptake or ingestion. This inhibition of antigen catabolism was associated with a corresponding inhibition of the development of T cell binding. Since inhibition of protein degradation by ammonia is thought to result from an increase in lysosomal pH, thereby inhibiting the action of acid hydrolases (26, 27), and/or by inhibition phagosome-lysosome fusion (28), these results implicate the lysosome in an intracellular pathway of antigen handling relevant to T cell-antigen recognition.

Finally, we examined the possibility that I-region gene products might be involved in handling of antigen by macrophages either by serving as receptors for the initial uptake of antigen or as molecules functioning in antigen catabolism. The inability of anti-Ia antibodies to alter the uptake or catabolism of antigen by macrophage populations highly enriched in Ia-positive cells (Table III) argues against such I-region gene involvement in initial antigen-handling events. Thus, I-region gene products may function either as receptors for fragments of antigen derived from antigen catabolism and/or as structures recognized directly by T lymphocytes.

The pathway of immunologically relevant antigen handling may be envisioned as follows: Antigen interacts with the macrophage cell surface directly through trypsin-sensitive receptors (29) or by way of FC/C3 receptors and is interiorized within phagosomes. Antigen-containing phagosomes then fuse with lysosomes, and partial degradation occurs. Some small fragments of antigen created by action of lysosomal proteinases are then released and transferred to the macrophage cell surface by a process akin to exocytosis (30). At some point after catabolism, the antigen fragments may cooperate with and/or interact with Ia molecules and thus form a multi-molecular antigenic structure recognized by T cells (3, 4, 31, 32). In contrast to the receptor initially involved in antigen binding, the putative membrane form of this processed antigen like Ia is relatively trypsin insensitive (23; Ziegler and Unanue, unpublished observations). It is not known whether the intracellular handling of antigen or the putative association of antigen fragments with Ia molecules is the rate-limiting step in antigen processing. In this regard, it would be of interest to determine whether antigens of minimal size, such as the octapeptide studied by Thomas et al. (33), would show a requirement for an antigen-processing period and ammonia effects as described here for a complex bacterial antigen. It is possible that the intracellular pathway of antigen handling may be bypassed by direct interaction of antigen fragments with macrophage cell surface Ia molecules. It should be noted that studies of the interaction of macrophage-bound molecules in B cell-T cell interactions have provided evidence for macrophage-associated molecules, but which retained their native structure and sensitivity to trypsinization (15, 34). Thus, the macrophage apparently serves through 2 distinct pathways of antigen handling to present antigen to both major sets of lymphocytes (2, 34).

The survival values of antigen degradation in the handling of pathogens may be viewed as a way to increase the number of different structural moieties that can serve as antigens. This advantage may be particularly relevant with regard to complex intracellular pathogens such as Listeria monocytogenes. Thus, bacterial components normally sequestered in the interior of organisms could conceivably serve as antigens, and the multiplicity of such antigenic determinants would make it less likely that a nonresponder status with respect to I-region gene function would be generated.

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The ability of rat leukocytes to hydrolyze a radiolabeled, surface-bound protein substrate in a solid phase assay was determined, and various factors that influence the process were measured. Unstimulated leukocytes hydrolyzed very little substrate. When the cell suspension was mixed with zymosan particles or incubated with preformed immune complexes, the amount of substrate hydrolysis increased dramatically. Not surprisingly, immune complexes at equilibrium proved to be the most effective in eliciting the response. Immune complexes attached to the surface along with the protein substrate were able to effectively induce hydrolysis, though they were not as effective as immune complexes in suspension. Three protease inhibitors, a,2-antitrypsin, a,1-macroglobulin, and soybean trypsin inhibitor, which were able to neutralize nearly all of the protease activity in rat neutrophil lysates, were tested for their ability to inhibit immune complex-induced protein hydrolysis. It was found that when the inhibitors were surface bound along with the substrate protein, they were effective in preventing the neutrophils from hydrolyzing the protein. However, when the same inhibitors were present in the fluid phase, they were much less effective. The relative inexeffectiveness of fluid phase protease inhibitors to block the protease activity of contact-activated leukocytes may explain how immune complex injury can take place in the presence of high concentrations of serum inhibitors.

Much interest in recent years has been generated in the study of the pathogenesis of immune complex-mediated tissue injury. Perhaps the simplest model of an immune-complex lesion is the Arthus reaction, which is a localized necrotizing vasculitis, with the antigen and antibody complexing within the vessel wall (1). Earlier studies have shown that the tissue injury seen with the Arthus reaction is complement and neutrophil dependent, with abolition of either abolishing the tissue injury (2).

Speculation has therefore centered in recent years on what constituents of neutrophils are responsible for the tissue injury. Studies done with neutrophil lysates or, more specifically, lysates of cytoplasmic lysosomal granules, reveal that they are capable of damaging basement membrane preparations in vitro (3). In rabbit neutrophils the specific lysosomal constituents responsible were found to be cathepsins D and E, in human neutrophils, neutral proteases (4). Many other studies (5, 6) have shown that lysosomal enzymes are released into the extracellular fluid during events such as phagocytosis of immune complexes. Therefore, a plausible explanation of the tissue injury seen in the Arthus reaction is the release of neutral proteases from the neutrophils during phagocytosis of the immune complexes in the vessel wall. It would therefore follow...