Antigen-Specific T Cell Cluster Formation on Antigen-Pulsed Macrophage Monolayers in Mice

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Abstract We describe the quantitative measurement of antigen-specific clusters formed by antigen-pulsed macrophages and immunized T cells in mice. We have found the peripheral blood T cells show very little non-specific adhesion to macrophages in mice. By using this population of lymphocytes in the peripheral blood as the source of immunized T cells, we could quantitate antigen-specific cluster formation. On OVA-pulsed monolayers of peritoneal exudate macrophages from normal BALB/c mice, syngeneic peripheral blood T cells from donors immunized with the same antigen develop 20-40 clusters per 1,000 macrophages, whereas the same T cells on non-pulsed monolayers develop only 0-5 cluster-like accumulations of cells. On antigen-pulsed monolayers of macrophages from allogeneic (C57BL/6 or A/J) mice, clusters are developed only in the negative range (0-5/1,000 macrophages). Considering the observation by Braendstrup et al, these data seem to suggest that histocompatibility between macrophages and T cells is required to develop antigen-specific T cell clusters on antigen-pulsed macrophage monolayers, and that the genetic restriction of immune responsiveness may be directly expressed in this initial form of cellular interaction between antigen-bearing macrophages and specific T cells.

It is well established that T cell dependent antigens are taken up (23), processed to peptide fragment (10), and then presented to T cells in the context of self class II major histocompatibility complex (MHC) molecules (31) by macrophages (Mφ).

The presentation of antigen to T cells by macrophages has been shown most definitively in the induction of specific helper T cells (11); Mφ presentation of antigen may also be important in the induction of cytotoxic T cells (36) and even of suppressor T cells (28). It appears that the presentation of antigenic information

Abbreviations: ABA, azobenzenearsonate; DNP, dinitrophenyl; Mφ, macrophages; MSA, mouse serum albumin; OVA, hen egg albumin; PEC, peritoneal exudate cells; PLC(T), peripheral blood lymphocytes T cell enriched fraction; Tyr, tyrosine.
by macrophages to T cells involves direct contact of the plasma membranes of the T cell and MΦ (27). This has been demonstrated in many laboratories by analyzing responses of T cells to antigen-pulsed macrophages, by means of estimating the induction of DNA synthesis or of resulting antibody production in vitro (18). In this interaction, T cells recognize not only the antigenic fragments held on the macrophage surface but also simultaneously recognize MHC products expressed on the antigen-presenting cells (24).

Another approach to analyzing the cellular interactions between macrophages and T cells has been morphological observations (1). Both antigen non-specific (37) and antigen-specific contact (16) between T cells and macrophages have been reported. Working with guinea pigs, Nielsen et al (20) have found that immunized T cells are bound specifically to antigen-bearing macrophages, and they also described the unique morphological characteristics of antigen-specific ‘clustering’ of immunized T cells on antigen-pulsed macrophages. However, in the mouse, in which much more analyzed genetic background is available, this antigen-specific cluster formation has not been reported so far. This is because the peripheral lymph node cells or thymocytes which have been used for the T cell source for cluster formation in guinea pigs exert strong non-specific adherence to macrophages in mice, and therefore, by applying the same experimental procedures as with guinea pigs, antigen-specific clusters are hardly visible in mice.

We have observed that the T cells from the peripheral blood of mice adhere very little to peritoneal exudate macrophages non-specifically. In this report, firstly we will establish the basic conditions for the quantitative observation of antigen-specific cluster formation of primed T cells on antigen-pulsed macrophages in mice by using the peripheral blood T cells as the source of immunized T cells. And secondly, we will demonstrate the necessity of concordance of histocompatibility between macrophages and T cells which participate in the formation of antigen-specific clusters. This suggests that one of the critical genetic restrictions of immune responses can be expressed as the possibility of formation of antigen-specific clusters between macrophages and T cells in mice.

**MATERIALS AND METHODS**

*Animals.* Mice of BALB/cAnN and C57BL/6 strains were from Charles River Japan Ltd., Tokyo, C3H/HeN and A/J strains were from Omura Experimental Animal Laboratories Co., Ltd., Kanagawa, Japan. Until used for the experiment, all mice were allowed food and water ad libitum in our SPF controlled animal rooms.

*Antigens.* Five-times crystallized hen egg albumin (OVA) was purchased from Seikagaku Kogyo Co., Ltd., Tokyo. 2,4-Dinitrobenzene sulfonic acid was purchased from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. Dinitrophenyl (DNP) ligand was coupled to OVA or mouse serum albumin (MSA) (Miles Biochemicals Laboratory Inc., Naperville, Ill., U.S.A.) according to the method of Eisen et al (9). These conjugates had 9.8 DNP groups per mol OVA and 9.7
DNP groups per mol MSA as calculated from molecular absorption assay. Azo-benzenearsonate (ABA) ligand was coupled to tyrosine (Sigma Chemicals Co., St. Louis, Mo., U.S.A.) according to the method described elsewhere (35).

**Immunization.** Mice were immunized with 100 μg/0.1 ml solution of antigens in Bacto-Adjuvant Complete H37 Ra (Difco Laboratories, Detroit, Mich., U.S.A.) in footpads, 8 days (unless otherwise stated) before they were sacrificed.

**Antigen-pulsed macrophage monolayers.** A half ml of mineral oil Marcol 52 (Esso Standard Oil Co., Ltd.) was injected into mice intraperitoneally 4 days before harvest. Animals were killed by cervical dislocation. The peritoneal cavity was rinsed well with 5–10 ml of Hank's balanced salt solution (HBSS: Nissui Seiyaku Co., Ltd., Tokyo) containing 10 U/ml of heparin (Novo Industries, Bagsvaerd, Denmark). Red cells were removed by lysis in isotonic ammonium chloride solution. Peritonealexudate cells (PECs) were cultured in HBSS with 5% FCS in 8-chamber culture slides (Lab-Tek Products, Miles Laboratories, Inc., Naperville, Ill., U.S.A.) for 7–9 hr at 37°C in humidified 5% CO₂ air, with or without appropriate antigen for the last 2 hr; 0.4 ml of PECs at 8×10⁵/ml were cultured per chamber. After incubation, chambers were washed three times with medium to remove excess antigen as well as the PECs non-adherent to the glass bottom. The remaining monolayer would comprise more than 95% spreading glass-adherent cells, and is referred to as antigen-pulsed macrophage monolayers.

**Peripheral blood T lymphocytes PLC(T).** Mice were bled at an appropriate time after immunization by heart puncture. White cells were separated from whole blood by density gradient centrifugation in Ficoll-Conray solution. (Ficoll, Pharmacia Fine Chemicals, AB, Uppsala, Sweden; Conray 400: Sodium 5-ace-tamidine-2,4,6-triiodo-N-methyl-isophthalamate, Daichichi Seiyaku Co., Ltd., Tokyo). White cell preparations were then applied to nylon wool columns to obtain the T cell enriched fraction. Nylon wool column passed cells were 93–95% sensitive to treatment with anti-Thy 1.2 antibody and complement. The viability of this T cell enriched fraction was >96% with trypan blue dye exclusion test. PLC(T) was suspended in RPMI1640 medium (Nissui Seiyaku) supplemented by 5% FCS (Gibco, Grand Island, N.Y., U.S.A.), 10 mM HEPES (Nissui Seiyaku), 0.03% L-glutamine (Seikagaku Kogyo), 0.05 mM 2-mercaptoethanol, 0.001% gentamycine and penicilline.

**Cluster formation.** 0.4 ml at 2.4×10⁶ cells/ml suspension of PLC(T) were added on the antigen-pulsed monolayer in each chamber. After an appropriate period of co-culture at 37 C in humidified 5% CO₂ air, non-attached ‘free’ lymphocytes were washed out. The remaining cells were fixed with 2% glutaraldehyde, stained by 4% Giemsa solution (Wako Pure Chemical Industries Ltd., Osaka, Japan) including a slight amount of acetic acid, and mounted for light microscopic observation.

**Estimation of clusters.** Clusters that satisfy the following criteria were considered to be specific and differentiated from non-specific attachment of lymphocytes to macrophages (see also Fig. 5).

1. Three or more lymphocytes must be attached to one macrophage.
Such attached lymphocytes must be located in one region of a macrophage.

Clustering lymphocytes must be composed of one ‘central’ lymphocyte and several ‘peripheral’ lymphocytes which are seen to attach to the ‘central’ one. This form of clustering lymphocytes is the same as that seen in guinea pigs and described by Braendstrup and Werdelin (4).

Most of the clustering lymphocytes, including the central cell, must be on the macrophage body.

For one test sample, i.e., one chamber of a slide, 700–1,400 macrophages were observed and counted, and the number of clusters was expressed as the mean±S.D. per 100 macrophages in three or more samples.

RESULTS

Non-Specific Adherence of Mouse Lymph Node Cells to Peripheral Exudate Macrophages

In the reported cluster formation experiments so far, the source of immunized T cells of guinea pigs was either lymph node cells or peritoneal exudate lymphocytes (15). In mice, to attempt similar cluster formation, we added lymph node T cells to the macrophage monolayer. After 12 hr of co-culture, the cells that were non-adherent to the monolayer were washed out. Residual cells were fixed and stained for light microscopic observation (Fig. 1A). Mouse lymph node T

Fig. 1. A, Prominent and strong non-specific adhesion of murine (BALB/c) lymph node lymphocytes (T-enriched) to syngeneic macrophages. B, Murine (BALB/c) peripheral blood T lymphocytes exert little non-specific adhesion to normal macrophages. Fixed and stained after 5 hr of co-culture (A, B). ×200 (A, B)
cells adhered non-specifically to every macrophage. It seemed very unlikely that one could identify antigen-specific clusters using this source of lymphocytes.

Lack of Non-Specific Adherence of Mouse Peripheral Blood T Cells to Peritoneal Exudate Macrophages

When the T cell enriched fractions of mouse peripheral blood cells were used, we found little non-specific adhesion to macrophages (Fig. 1B). Normal peritoneal macrophages and normal (non-immunized) peripheral blood T cells also showed little non-specific adhesion under the culture conditions described (Fig. 1).

The Effect of the Dose of Peripheral T Cells on Non-Specific Adhesion to Normal Macrophages

To standardize the conditions of this background reaction of non-specific adhesion, the dose response correlation between the number of peripheral T cells added and the number of cells which adhere non-specifically to normal macrophages was examined (Fig. 2). Using a concentration of peripheral blood T cells two-

![Fig. 2. Dose-dependent profile of non-specific attachment of T cells to constant number of macrophages. Numbers of attached PLC(T)s per 100 Mφs are plotted to the added PLC (T)s: Mφs ratio. Mφ concentration is always a constant of $8 \times 10^5$/ml x 0.4 ml/well. 100-200 PLC(T)s are attached to 100 Mφs (almost 1:1 non-specific adhesion), when 2-3-fold concentration of PLC(T)s (i.e., $1.6-2.4 \times 10^6$/ml x 0.4 ml/well) are added to macrophage-monolayers. Mice, BALB/c; co-culture period, 10 hr. Mean values of data (M) in three independent experiments are plotted ○, standard deviation (S.D.) are shown by ○—○ vertical lines.](image-url)
to threefold higher than that of the input peritoneal exudate cells, non-specific adhesion of almost one lymphocyte to one macrophage has been observed. The fewer T cells added, the less non-specific adhesion was observed, but at the same time the number of specific clusters also declined. On the other hand, if the T cell: PEC ratio was above 2-3-fold, it was difficult to differentiate at least some of the specific clusters from non-specifically formed cell groups.

The Appropriate ‘Background’ Non-Specific Adhesion of Lymphocytes to Observe Antigen-Specific Cluster Formation

Figure 3A shows an actual antigen-specific cluster of lymphocytes and the ‘background’ non-specific adhesion of lymphocytes under the condition of 3:1 peripheral blood T cell to PEC. Antigen-specific clusters of petal shape accumulation of cells (Fig. 3B) was clearly distinguishable from the non-specific adhesion of randomly distributed cells. Thus, the T cell: PEC ratio used in the following experiments was fixed at 3:1.

Time-Dependent Appearance of Peripheral Blood T Cells Which Could Form Clusters

Emergence of antigen-specific T cells into the peripheral blood stream after immunization is known to follow a characteristic time course, having an initial stage of accumulation of such T cells in the draining lymph nodes to give a lag phase of

![Figure 3](image-url)
emergence (32). While in our cluster formation assay, the T cells that form clusters effectively were expected to emerge into peripheral blood in a similar time-course fashion, peripheral blood samples of 4–20 days after immunization with OVA were tested. Figure 4 shows that peripheral blood taken 8 days after immunization contained T cells that give rise to the most antigen-specific clusters. Thus, in the following experiments, peripheral blood samples were collected 8 days after immunization.

The Effect of Concentration of Antigen to Pulse Macrophage Monolayer in vitro on Cluster Formation

The effect of antigen concentration used to pulse macrophages in vitro was examined using OVA as a model antigen. OVA-specific clusters were developed at a final concentration of 1, 10, 25, 100, 200, 500, 1,000, and 2,000 μg/ml of antigen. In most experiments, significant cluster formation was observed at antigen concentrations above 10 μg/ml (data not shown). From the typical dose response curve shown in Fig. 5, the optimal antigen concentrations were considered to be 100–200 μg/ml. In the following experiments, 100 μg/ml of other antigens were also used to pulse macrophages and in all cases we observed as good cluster formation as was observed using OVA. Furthermore, this optimal concentration of antigen was comparable with the antigen concentrations used to pulse macrophages in vitro in many laboratories (26).
Time-Dependent Development of Cluster Formation

Development of cluster formation after the addition of peripheral blood T cells to antigen-pulsed macrophage monolayers was examined at 2, 12, and 20 hr of incubation. After 12 hr of co-culture, antigen-specific clusters were observed to reach optimal levels (Table 1).

Antigen Specificity of Cluster Formation

Significant cluster formation could not be observed on the macrophage mono-

Table 1. Incubation time-dependent change of number of clusters per 1,000 macrophages

<table>
<thead>
<tr>
<th>Incubation period</th>
<th>(A) Antigen non-pulsed Mφs</th>
<th>(B) Antigen pulsed Mφs</th>
<th>(B)/(A) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 hr</td>
<td>1.95 ± 1.95</td>
<td>4.75 ± 1.25</td>
<td>2.44</td>
</tr>
<tr>
<td>12 hr</td>
<td>5.39 ± 5.39</td>
<td>43.89 ± 24.60</td>
<td>8.14</td>
</tr>
<tr>
<td>20 hr</td>
<td>7.22 ± 4.58</td>
<td>32.64 ± 8.02</td>
<td>4.12</td>
</tr>
</tbody>
</table>

Mφs and PLC(T)s are all from C57BL/6 mice. Pulsing antigen is ABA-tyrosine. Mean values of data in three or more experiments (M) ± standard deviations (S.D.) are shown.
layer that had not been pulsed with specific antigens even after an appropriate period of co-culture (Table 1), although these macrophage monolayers were considered to be pulsed with unrelated proteins which exist in supplemented FCS. In addition, the peripheral blood T cells from non-immunized animals formed no significant clusters on antigen-pulsed macrophage monolayers (data not shown). Immunization or pulsing of monolayer with irrelevant antigen also results in a loss of significant cluster formation. In fact, the ratios of %o clusters of ABA-tyrosine specific PTL, and DNP-MSA specific PTL on OVA-pulsed macrophage monolayer to those on antigen-nonpulsed macrophage monolayer were 1.23 and 0.82, respectively. Therefore, cluster formation of immunized T cells on antigen-pulsed macrophage monolayer was antigen specific.

**Cluster Formation by Several Antigens**

Cluster formation was not a special phenomenon observed only in the case of OVA (Table 2). Clusters were formed specifically also with ABA-Tyr or DNP-MSA. ABA-Tyr is known to evoke delayed-type hypersensitivity in guinea pigs (2) and in rats (6). In mice ABA-coupled syngeneic cells can trigger ABA specific delayed-type reactions (3). Therefore, the significant cluster formation with nylon wool column passed peripheral blood cells from ABA-Tyr immunized mice suggests that the major subpopulation of lymphocytes participating in cluster formation would be thymus-derived (T) cells.

**Cluster Formation in Several Strains of Mouse**

Antigen-specific cluster formation was observed not only in a particular strain but in all of the several strains of mouse tested, provided the antigen-pulsed macrophages and immunized peripheral T cells were syngeneic (Table 3).

**MHC Restriction in Cluster Formation**

We examined further if there exists a genetic restriction between macrophages and T cells in cluster formation. Immunized peripheral T cells could not form significant clusters on antigen-bearing macrophage monolayers allogeneic to T cells (Table 4). Furthermore, to inhibit antigen-specific cluster formation, the difference

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### Table 2. Formation of antigen-specific clusters with several antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>%o Clusters on</th>
<th>(B)/(%A) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A) Antigen</td>
<td>(B) Antigen</td>
</tr>
<tr>
<td></td>
<td>non-pulsed Mφ</td>
<td>pulsed Mφ</td>
</tr>
<tr>
<td>OVA</td>
<td>2.10 ± 2.97</td>
<td>15.39 ± 4.32</td>
</tr>
<tr>
<td>OVA</td>
<td>7.20 ± 10.18</td>
<td>25.10 ± 22.70</td>
</tr>
<tr>
<td>ABA-tyrosine</td>
<td>2.65 ± 0.95</td>
<td>13.48 ± 3.57</td>
</tr>
<tr>
<td>DNP-MSA</td>
<td>12.57 ± 8.75</td>
<td>30.20 ± 4.57</td>
</tr>
</tbody>
</table>

Mφs and OLC(T)s are all from BALB/c mice. The period of Mφ-monolayer, lymphocytes co-culture is 12 hr. M±S.D. as in Table 1.
in MHC need not include the whole H-2 region. The results of experiment 4 in Table 4 suggest that the difference of the left half of H-2, including I-A subregion (to say nothing of any other non-H-2 differences) can sufficiently prevent the formation of antigen-specific clusters.

**DISCUSSION**

Antigen-specific cluster formation of immunized T cells on antigen-bearing macrophage monolayers has been observed in the guinea pig system (25). However, in mice, due to the non-specific adhesion of lymphocytes to macrophages, the formation of antigen-specific clusters has been very difficult to observe. In this report we have established a well-defined technique of antigen-specific cluster formation in mice whose well-characterized genetic backgrounds will enable us to better analyze the genetic regulation of antigen-specific T cell and macrophage cluster formation. In our method of cluster formation, it is critically important to use peripheral blood T cells as the source of immunized T cells in order to observe
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antigen-specific clusters quantitatively. It has been observed that lymphocytes in
the circulation show little non-specific adhesion to macrophages or cells of the
reticuloendothelial system by using thoracic duct cells in rat (33) or peripheral
blood cells of man (21). We have demonstrated that in mice as well, peripheral
blood T cells do not adhere to normal macrophages non-specifically, whereas
lymph node T cells do (Fig. 1, A and B and Fig. 2). Using this population of
peripheral T cells as the source of immunized T cells, we could observe antigen-
specific cluster formation in mice quantitatively in the characteristic fashion which
is dependent on antigen dose, days after immunization, and on hours of co-culture
(Fig. 3, A and B, and Figs. 4 and 5).

Two cells are involved in the cluster formation; the cells that bear and present
antigens are from the glass-adherent population of peritoneal exudate cells which,
after 7 to 9 hr of incubation, comprise over 95% of the ‘spreading’ cells. On the
other hand, immunized lymphocytes that form clusters on the adherent cell mono-
layer appear to be a T cell population, as we have used T cell enriched nylon wool
column non-adherent fraction of peripheral blood lymphocytes as an immunized
lymphocytes source. This supposition is supported indirectly by the data of experi-
ments using ABA-Tyr as antigen. Although, both ‘macrophage’ and ‘T cell’
enriched fractions may contain a small amount of B cells as well, MHC restricted
manner of cluster formation argues against the possibility of B cell clustering. Yet
we will not be surprised if any B cells can stick to the antigen-presenting cells spe-
cifically. We suspect such a binding may not form a typical clustering as shown
in Fig. 3 B.

To form significant clusters the concordance between the antigen used to pulse
macrophages and that used to immunize animals which provide the T cell source
is essential (12–14, 34). This suggests that cluster formation is antigen specific
(Table 1). Furthermore, this cluster formation is a general phenomenon, observed
with several antigens and in several strains of mice that are able to respond to those
antigens (Tables 2 and 3). The requirement for histocompatibility between T cells
and macrophages for the transmission of antigenic information between these two
types of cells has been classically established in both the proliferative response of T
cells (29) and the increase of plaque-forming B cells induced by T cells which have
been stimulated by antigen with macrophages (22). In the induction of helper T cells
by T-dependent antigens, it is known that the I-A subregion of H-2 must be shared
between antigen-presenting cells and T cells (38). In cluster formation in guinea
pigs, Ia antigens seem to play an important role in the physical association of T cells
and macrophages (5). Our finding in this report of a requirement of histocom-
patibility in cluster formation in mice is consistent with the findings in the guinea
pig system.

T cells recognize antigens in the context of MHC gene products that are ex-
pressed on the surface of antigen-presenting cells (30). However, whether such
recognition by T cells is mediated by two configurationally separated structures of
recognition unit(s) (dual recognition) (8) or by a single continuously provided
structure of recognition unit of T cells (modified self) (17) is, in spite of recent
progress of molecular studies on T cell receptors (7), still unclear. Both of these arguments stand, although being considerably modified from their original forms.

It is not clear why PTL failed to form clusters on antigen-pulsed allogeneic macrophage monolayers. However, these results may be explained by the following hypothesis: circulating lymphocytes after immunization are mainly T cells specific for the immunizing antigen, or their affinity to antigen-pulsed allogeneic macrophage monolayers is much lower than that to antigen-pulsed syngeneic macrophage monolayers.

We also do not know for sure what are the repertoire size of T cells recognizing self determinants and allo determinants. It is possible that some of the T cells in the cluster may not be antigen specific but self-specific.

Taking the highly complicated structure of activated T cells receptor complex into account, we can further assume that the recognition mechanisms of the primed antigen-specific T cells and of the primary unprimed allo-reactive T cells may be quite different. In fact, in the course of activation of normal T cells and of established cloned T cells, CD4 molecule seems to behave differently, therefore probably resulting in the different composition of active T cell receptor complex. (Saizawa et al, submitted for publication).

Many of these interesting questions are still unresolved, yet, using the well-defined clonal source of T cells, attempts to ‘visualize’ the roles of every cell interaction molecule (here we include T cell receptor as well) at the cluster level are underway. Finally we would like to emphasize the importance of our finding of the lack of non-specific adherence of peripheral blood T cells to macrophages, which, we consider, must be closely related to the expression and regulation of lymphocyte homing receptors.

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