INTERACTIONS BETWEEN T HELPER CELLS AND DENDRITIC CELLS DURING THE RAT MIXED LYMPHOCYTE REACTION

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T helper (Th) cells are stimulated into cell division by the Ia+ dendritic cell (DC) in the allogeneic mixed lymphocyte reaction (MLR) (1–3). The Th cells recognize class II antigens predominantly and produce interleukin 2 (IL-2). These cells require the DC as a specialized accessory cell, in contrast to cytotoxic T cell precursors, which can be driven to effector function by any type of allogeneic stimulator cell in combination with IL-2 (2). 1–2 d after the initiation of the MLR, small clusters are seen in culture, made up of T blasts and DC. Clusters also arise during the syngeneic MLR and in primary antibody responses in vitro (3–5). These clusters are important functionally since the clustered T cells produce IL-2, whereas the nonclustered cells do not; in addition, it is the clustered T cells that proliferate.

This paper describes the cell-cell interactions between Th cells and DC in the rat MLR in an attempt to determine their molecular basis. DC have previously been isolated from lymph node, thymus, or spleen but the procedure is lengthy and the yield of DC per rat is low (6). After mesenteric lymphadenectomy (MLNX) of rats, cells with the characteristics of DC appear in the thoracic duct lymph (7) which are potent stimulators of the primary allogeneic MLR (1). MLNX thoracic duct lymph has been used in the present study as a source of DC.

During the MLR, T blasts appear after several days of culture and proliferation peaks at about day 4–6. The blasts then return to a resting state and proliferation ceases. These resting T cells (termed memory cells) can then be restimulated in a secondary MLR; the interactions of these cells and Th blasts with DC will be described.

W3/25 monoclonal antibody (mAb) labels rat thymocytes, Th cells, and macrophages (8–10). It recognizes a surface glycoprotein of M, 48–52,000 and

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Abbreviations used in this paper: BSA, bovine serum albumin; Con A, concanavalin A; DAB, Dulbecco’s A + B medium; DC, dendritic cell; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FcR, Fc receptor; IL-2, interleukin 2; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLNX, mesenteric lymphadenectomy; MLR, mixed lymphocyte reaction; Mφ, macrophage; PBS, phosphate-buffered saline; RAM, rabbit anti-mouse (γG (F(ab’))2 fragments); RAM-FITC, RAM coupled to fluorescein isothiocyanate; SRBC, sheep red blood cells; TdR, thymidine; Th cell, T helper cell; Tc/s cell, T cytotoxic/suppressor cell.
is also present on macrophages (11). W3/25 antigen is equivalent to the human T4 antigen, which is now called CD4, and to mouse L3T4 (12-14). W3/25 inhibits proliferation in the MLR and does so by acting on the responding T cells (1, 15). We examined the effects of W3/25 and MRC OX-6 (anti-rat class II antigens) on cell interactions and proliferation of resting Th cells, Th blasts, and Th memory cells. The results suggest that the molecules recognized by these antibodies are involved in antigen-specific cell interactions.

Materials and Methods

Inbred rats PVG-RT1\(^{a}\), PVG-RT1\(^{b}\) (AO), and DA-RT1\(^{a}\) (referred to as HO, HOB2, and DA, respectively), and F1 hybrids between HO and HOB2 were obtained from the Specific Pathogen Free Unit of the MRC Cellular Immunology Unit, Oxford.

Antibodies. Mouse mAb were in the form of either tissue culture supernatant from cloned cells or immunoglobulin (Ig) purified from ascites (16). The mAb used were W3/25 (labels thymocytes, Th subset, and macrophages [8-10]); W3/13 (labels thymocytes, T cells, polymorphs [8]); MRC OX-6 (anti-rat MHC class II antigens [I-A] [17]); MRC OX-8 (labels thymocytes, cytotoxic-suppressor T cells [Tc/s], and natural killer cells [18, 19]); MRC OX-12 (anti-rat immunoglobulin k chain preference for k allotype [20]); MRC OX-17 (anti-rat major histocompatibility complex [MHC] class II antigen [I-E] [21]); MRC OX-18 (anti-rat MHC class I antigens [21]); MRC OX-19 (pan T [22]); MRC OX-21 (anti-human C3b inactivator [23]); MRC OX-22 (anti-rat leukocyte-common antigen; labels Th subset, Tc/s, and B cells [24]); MRC OX-26 (anti-rat transferrin receptor [25]); MRC OX-34 (pan T [11]). F(ab')\(_2\) fragments were prepared from W3/25 and MRC OX-6 IgG using methods described previously (16). Other antibodies used were rabbit F(ab')\(_2\) anti-mouse IgG (RAM); RAM conjugated to fluorescein isothiocyanate (RAM-FITC) (9), and RAM conjugated to horseradish peroxidase (10).

Th Cells. HO thoracic duct lymphocytes (TDL) were collected overnight at 4°C into Dulbecco's A + B medium (DAB) containing 20 U/ml heparin. W3/25 + Th cells were prepared from TDL by removing MRC OX-8 + Tc/s cells and MRC OX-12 + B cells using rosette depletion (26).

Th cell blasts were generated in a primary MLR between HO W3/25 + responding T cells and F1 spleen cell stimulators. The spleens were removed aseptically, teased apart, and filtered through cotton wool. The cells were then washed twice in DAB containing 0.2% bovine serum albumin (DAB/BSA). The MLR was set up in RPMI 1640 medium containing antibiotics, 2.5 mM 2-mercaptoethanol, and 5% DA serum. 10\(^5\) W3/25 + responding T cells and 5 \times 10\(^5\) F1 irradiated spleen cells were cultured in 0.2 ml medium per well in sterile 96-well U-bottomed microtiter plates (Sterilin, U.K.) at 37°C in a humidified incubator, with 5% CO\(_2\). Several plates were set up and, after 5 d culture, the cells were harvested and dead cells removed by centrifugation through Isopaque-Ficoll at 700 g for 30 min at 25°C. The viable cells were restimulated using 2 \times 10\(^5\) responders/ml and 2 \times 10\(^6\) F1 irradiated spleen cells/ml in 20 ml RPMI 1640 containing 10% fetal calf serum (FCS) in a Falcon flask. This cell line was then subcultured as above every 2–3 d. Stimulators were given 1,500 rad irradiation from a \(^{137}\)Cs source at 100 rad/min. Th memory cells were generated in a primary MLR set up as described above and the cells were harvested after 10–12 d culture, when proliferation had stopped.

The proliferation of cultures was assayed by pulsing each well with 0.5 \(\mu\)Ci (18.5 kBq) of \(^{3}H\)thymidine (\(^{3}H\)TdR) (Amersham International, Amersham, England) 18 h before the end of the incubation period. Uptake of radioactivity was determined after harvesting onto glass fiber filter paper (Whatman Ltd, Maidstone, England). Results are expressed as the arithmetic mean of quadruplicate cultures ± standard deviation.

MLN X DC. DC were prepared from rats that had undergone mesenteric lymphadenectomy. The cecal, mesenteric, portal, and pancreatic lymph nodes were removed from 6-wk-old rats by blunt dissection and the animals were allowed to recover for at least 6 wk before use (7). The animals were irradiated (500 rad) using a \(^{137}\)Cs source of \(\gamma\) rays.
After thoracic duct cannulation cells were harvested from the first or second overnight collection and washed three times in phosphate-buffered saline (PBS)/0.2% BSA. They were resuspended in 5 ml PBS/BSA, layered over 2 ml 15% wt/vol Metrizamide (Nygard, Oslo) in PBS/BSA, and centrifuged at 600 g for 10 min. The low density cells at the interface were recovered and washed twice in PBS/BSA before use.

Macrophages (Mφ). Resident peritoneal cells were harvested in 15 ml of ice-cold PBS containing 20 U heparin/ml. Yields were ~10^7 cells per rat. These cells were washed in PBS, layered over 20% wt/vol Metrizamide, and centrifuged at 600 g for 10 min. The cells at the interface were washed twice before use and were enriched in Mφ. They were 85% Fc receptor-positive (FcR+), 5% Ia+, and 90% phagocytic (latex ingestion).

Splenic Mφ were prepared by centrifuging a spleen cell suspension on Isopaque-Ficoll for 30 min at 25°C. The cells at the interface were washed, suspended in RPMI 1640 containing 10% FCS at 2.10^7 cells/ml, and incubated on plastic petri dishes for 3 h at 37°C. The dishes were washed three times with warm medium and the adherent cells were removed with a rubber policeman. These cells were washed and spun on a 20% Metrizamide gradient as described above. The cells recovered from the interface were enriched in Mφ and centrifuged as before. The pellet of rosetted cells was recovered, the red cells lysed in NH4Cl, and the recovered cells were washed. They were 90% FcR+, 50-70% Ia+, and 85% phagocytic.

Other Cell Types. B cells were obtained by labeling TDL with MRC OX-12 followed by RAM-FITC and cell sorting on a FACS II (Becton Dickinson Immunocytometry Systems, Mountain View, CA). T and B concanavalin A (Con A) blasts were generated by stimulating spleen cells that had been cultured at 2 X 10^6 cell/ml in RPMI 1640 medium containing 5% fetal calf serum (FCS), for 3 d with 5 µg/ml Con A. After this time the cells were washed three times in 10 mg/ml α-methylmannoside to remove Con A. T blasts were obtained by removing B cells after labeling with MRC OX-12 and B blasts by removing T cells after labeling with MRC OX-8 and W3/25, followed by rosette depletion (26). After rosetting for 20 min at 4°C, the cell suspension was layered directly onto a 20% wt/vol cushion of Metrizamide and centrifuged as above. The nonrosetted cells remained at the interface and were washed twice before use.

Preparation of MLR Supernatant Containing IL-2 and IL-2 Assay. Supernatants containing IL-2 were obtained after culturing the Th blast cell line for 24 h using 5 X 10^5 responders ml plus 2 X 10^5 fresh stimulators/ml, and then removing the cells by centrifugation. To assay IL-2, T cell blasts were generated by culturing HO spleen cells at 2 X 10^6 cells/ml with 5 µg/ml Con A. Cells were harvested after 3 d and washed to remove Con A as described above. The supernatant to be tested was added at 1:2 and 1:4 dilutions to 4 X 10^4 blasts in a total volume of 0.2 ml RPMI containing 5% FCS, and proliferation was assayed after 24 h as described previously.

Clustering Assay. Th cell blasts or memory cells were incubated with 5 µg/ml fluorescein diacetate (Sigma Chemical Co., St. Louis, MO) in RPMI + 5% FCS for 30 min at room temperature after which the cells were washed and spun through PBS containing 10% BSA. These cells were then mixed with MLNX DC or other cell types in 0.1 ml of RPMI 1640 plus 5% FCS, containing 20 µg/ml DNase I (Sigma Chemical Co.), in an Eppendorf tube. The cells were incubated for 30 min at 37°C on a rotary shaker set at 100 rpm, after which the tubes were placed on ice. The number of fluorescent particles was determined using a hemocytometer on a fluorescent microscope and the percent aggregation was equal to the percent reduction in fluorescent particle number from time zero. All assays were done in triplicate.

Cell-Cell Binding Assay. Th memory cells were labeled with fluorescein diacetate and mixed with MLNX DC in 0.1 ml in an Eppendorf tube. The tubes were centrifuged at 250 g for 5 min at room temperature and the pelleted cells were incubated for 15 min at 37°C. The cells were resuspended by vigorous pipetting (12 times) using an Eppendorf pipette (P200) set at 0.09 ml. The resuspended cells were kept on ice before counting.
The number of fluorescent T cells that were free or bound to DC were counted using a hemocytometer on a fluorescent microscope. The percentage of T cells bound to DC was then calculated.

Miscellaneous. Labeling of cells with mAb and analysis or separation of cells on the FACS II were performed as described previously (9). Cytocentrifuge preparations of cells were stained using the immunoperoxidase technique (10).

Results

Isolation of MLNX DC. MLNX TDL from irradiated rats contained 1–5% DC from the first overnight collection and up to 20% DC from the second overnight collection as judged by morphology. The DC were enriched to 70–90% purity after centrifugation of the MLNX TDL on the metrizamide gradient; between $5 \times 10^5$ and $2 \times 10^6$ DC could be obtained per rat. Fig. 1a shows a cytocentrifuge preparation of enriched DC labeled with MRC OX-6 and stained by the immunoperoxidase technique. Most of the cells have the irregular morphology typical of DC and they are strongly Ia*. These purified cells were not phagocytic and lacked Fc receptors. Up to 50% of DC could be labeled with W3/25 mAb (not shown).

Stimulator Cell Requirements for Proliferation of Unprimed Th Cells, Th Blasts, and Th Memory Cells. Unprimed Th cells, Th cell blasts, and Th memory cells were prepared as described above. The Th blasts were actively dividing cells, whereas the Th memory cells had reverted back to a resting state. The cell type required for stimulation of unprimed Th cells and for restimulation of Th cell blasts and Th memory cells was tested (Table I). The Th cells were incubated with allogeneic spleen cells or purified DC, Mϕ, or B cells. The results showed that the DC is the major stimulating cell for all three stages of differentiation of the Th cell and is up to 100× as potent as whole spleen cell populations. Ia+ Mϕ were very poor stimulators but B cells did have some capacity to restimulate Th blasts and Th memory cells. The antigen specificity of the Th blasts and Th memory cells generated in the MLR was tested by incubating them with third-party (DA rat) MLNX DC. Only a small proliferative response was induced by these DC (Table I). Syngeneic DC (HO rats) similarly showed a very low stimulatory effect.

Surface Phenotypic Changes of Th Cells on Activation in MLR. Unprimed W3/25+ T cells and Th blasts were incubated with mAb followed by RAM-FITC, and the percentage of labeled cells was determined on the FACS II (J. Green and W. Jefferies, unpublished results). Virtually all (96%) of the activated Th cells were labeled by W3/25 and ~25% of these cells were also labeled with MRC OX-8, which does not label resting Th cells. MRC OX-22, which is on 71% of resting Th cells, disappeared on activation. The transferrin receptor (identified with MRC OX-26) was expressed on up to 80% of the Th blasts, but class II antigens (identified with MRC OX-6 and MRC OX-17) were not expressed on activation. The pan T markers W3/13, MRC OX-19, and MRC OX-34 were all retained after activation. Th memory cells had a similar phenotype to that of the Th blasts.

Formation of Clusters Between Th Cells and DC. When W3/25+ Th cells were incubated in culture with allogeneic DC, small clusters were formed after 1–2 d (Fig. 1c) and these have been shown to comprise T blasts and DC (3). The
Table 1

Proliferation of Th Cells Stimulated with Different Cell Types

| Stimulator cell type | Stimulator cell No. | [3H]Tdr uptake* (cpm x 10^-3) |
|----------------------|---------------------|-------------------------------|
|                      |                     | Primary MLR (2 x 10^5) | Th Blasts (2 x 10^4) | Th Memory Cells (2 x 10^5) |
|                      |                     | 2.0 ± 0.5 | 3.2 ± 1.1 | 1.6 ± 0.4 |
| Spleen               | 5 x 10^5            | 84.2 ± 6.8 | 57.3 ± 4.9 | 51.3 ± 3.6 |
|                      | 2 x 10^4            | 4.1 ± 1.4 | 6.9 ± 1.2 | 8.2 ± 1.2 |
| B                    | 10^5                | 6.1 ± 1.2 | 14.2 ± 2.6 | 14.0 ± 2.0 |
|                      | 2 x 10^4            | 4.5 ± 0.8 | 4.1 ± 0.6 | 3.6 ± 0.4 |
| Ia* Mφ                | 10^5                | 5.1 ± 1.3 | 5.2 ± 0.4 | 4.7 ± 1.2 |
|                      | 2 x 10^4            | 5.2 ± 0.9 | 3.9 ± 1.1 | 4.2 ± 0.4 |
| DC                   | 2 x 10^4            | 102.9 ± 6.2 | 64.6 ± 4.8 | 63.8 ± 5.0 |
|                      | 4 x 10^4            | 87.3 ± 4.9 | 13.6 ± 2.4 | 27.2 ± 0.9 |
|                      | 8 x 10^3            | 8.6 ± 2.0 | 4.1 ± 1.0 | 4.6 ± 1.1 |
| DC (DA rats)         | 2 x 10^4            | — | 8.4 ± 2.1 | 8.9 ± 1.4 |
|                      | 4 x 10^3            | — | 3.1 ± 0.4 | 4.1 ± 0.8 |

* Proliferation was assayed after 5 d incubation of primary MLR, and after 2 d incubation using Th blasts or Th memory cells. [3H]Tdr uptake was measured after an 18 h pulse before the end of the incubation period.

† W3/25 Th cells were isolated from HO TDL after removal of B cells and Tc/s cells by rosette depletion.

§ Th blasts and memory cells were derived from 5-d and 10-d MLRs, respectively, using F1 spleen cell stimulators. For proliferation assays, resting Th cells were used at 2 x 10^5 cells per well whereas Th blasts or memory cells were used at 2 x 10^5 per well in 0.2 ml RPMI 1640 + 5% DA serum. Stimulators were from HOB2 rats unless otherwise stated.

Fluorescence diacetate. Fluorescent Th cell blasts were incubated with a variety of cell types and the percent aggregation was determined. Table II shows that when Th blasts were cocultured with allogeneic MLNX DC there was a high level of aggregation. This did not occur when other cell types were incubated with the T cells. Unprimed Th cells did not aggregate in the presence of DC using this short-term assay (not shown). To test the antigen specificity of this cell-cell interaction between Th blasts and DC, cells were prepared from syngeneic (HO) and third-party (DA) rats and compared with allogeneic (HOB2) DC in the clustering assay. DC from the three rat strains induced clustering to the same extent (Table II) indicating that the aggregation is not dependent on an MHC difference between the cells. However, it is only the allogeneic DC used...
FIGURE 1. (a, b) Cytocentrifuge preparations of enriched MLNX DC stained by the immunoperoxidase technique (10): (a) labeled with MRC OX-6, (b) labeled with MRC OX-21 used as negative control. (c) MLR between HO W3/25 Th cells and HOB2 MLNX DC 48 h after initiation of the culture. (d) Clusters observed 3 h after incubation of $10^5$ HO W3/25 Th blasts with $2 \times 10^4$ HOB2 DC. (e) W3/25 Th blasts cultured for 3 h without DC. (a, b) $\times 500$; (c) $\times 100$; (d) $\times 200$, (e) $\times 100$. 
TABLE II

Aggregation Between Th Blasts and Memory Cells with Other Cell Types

| Exp. | Stimulator cells (2 x 10⁶) added to T cells (10⁵) | Percent aggregation of Th blasts | Percent aggregation of Th memory cells |
|------|-----------------------------------------------|----------------------------------|---------------------------------------|
| 1    | DC (allo)                                      | 6 ± 2                            | 5 ± 2                                 |
|      | MΦ (allo)                                      | 62 ± 5                           | 24 ± 4                                |
|      | IA+ MΦ (allo)                                  | 9 ± 2                            | 8 ± 3                                 |
|      | Peritoneal MΦ (allo)                           | 8 ± 2                            | 6 ± 2                                 |
|      | B cells (allo)                                 | 9 ± 2                            | 5 ± 2                                 |
| 2    | DC (allo)                                      | 7 ± 2                            | 4 ± 1                                 |
|      | MΦ (allo)                                      | 47 ± 5                           | 27 ± 5                                |
|      | DC (syng)                                      | 42 ± 4                           | 22 ± 2                                |
|      | DC (third party)                               | 41 ± 4                           | 23 ± 3                                |

In Exp. 1, 10⁵ fluorescently labeled HO Th blasts or memory cells were incubated alone or with a variety of allogeneic (HOB2) cell types. After 30 min at 37°C the number of fluorescent particles was counted and the reduction from zero time gave the percent aggregation.

In Exp. 2, the HO Th cells were incubated with DC isolated from allogeneic (HOB2) syngeneic (HO) or third-party (DA) rats. When unprimed HO Th cells were incubated with allogeneic DC, there was 6 ± 2% aggregation of the T cells; with syngeneic DC, there was 5 ± 2%.

TABLE III

Interaction Between Con A Blasts and DC

| Blasts (10⁵) | DC (2 x 10⁶) | Percent aggregation of blasts |
|-------------|--------------|------------------------------|
| T           | —            | 6 ± 1                        |
| T           | Syngeneic    | 45 ± 3                       |
| T           | Allogeneic   | 49 ± 5                       |
| B           | —            | 6 ± 2                        |
| B           | Syngeneic    | 31 ± 3                       |
| B           | Allogeneic   | 34 ± 3                       |

HO spleen cells were stimulated with 5 μg/ml Con A for 3 d. T and B blasts were obtained after rosette depletions with MRC OX-12 (T blasts) and W3/25 plus MRC OX-8 (B blasts). Aggregation of blasts with DC was determined as described in Table II.

as stimulators in the primary MLR that are able to restimulate these Th blasts to proliferate (Table I). Th memory cells formed small clusters with DC having the same properties as those formed by Th blasts (Table II).

**Interactions Between Con A-activated T and B Cells with MLNX DC.** T and B cell blasts were separated after stimulating HO rat spleen cells with Con A and the ability of DC to induce cluster formation with these polyclonally activated cells was tested. Table III shows that T and B blasts do not aggregate extensively unless they are cocultured with DC. The amount of aggregation was the same in the presence of either syngeneic or allogeneic DC.

**Interaction of Th Memory Cells with DC.** In a different assay to assess the binding of individual T cells to DC, the two cell types were pelleted together and vigorously resuspended after 15 min incubation. The T cells were previously
labeled with fluorescein diacetate and the percent of T cells bound to DC was
determined. The conditions used in this assay tend to dissociate weakly stable
clusters and, when Th memory cells were incubated with DC from different rat
strains, it was possible to detect antigen-specific binding between the two cell
types. For example, there was a much greater number of Th memory cells bound
to allogeneic (HOB2) DC than to either the syngeneic (HO) or third-party (DA)
DC (Table IV). When this assay was attempted using the Th blasts, either too
many aggregates still remained after dispersing the cell pellet or, with more
vigorous pipetting, only very few blasts remained bound to the DC. Thus, it was
not possible to obtain data for the T blasts in this assay.

Effects of W3/25 and MRC OX-6 on Proliferation of Th Cells. Previous data has
shown that W3/25 inhibits the primary MLR when added at the initiation of the
culture; both W3/25 and MRC OX-6 block the response of primed T cells to
soluble antigens (1, 15, 27). The effects of these two mAb on proliferation of
Th blasts and Th memory cells, restimulated with allogeneic cells or IL-2, was
tested and compared with the inhibition of the primary MLR. The results showed
that both antibodies inhibited the proliferation of Th cells in the primary MLR
and also blocked the secondary response of Th memory cells to allogeneic cells
(Table V). The inhibitory effects were obtained using F(ab')2 fragments of the
antibodies; it was necessary to use a higher concentration of antibody to block
the secondary as compared with the primary response. In contrast, the restimu-
lation of Th blasts with allogeneic cells in a secondary MLR was blocked by MRC
OX-6 but was unaffected by W3/25 mAb. To see the inhibitory effect with MRC
OX-6 it was necessary to reduce the number of DC (to $5 \times 10^5$ DC per well) to
give a suboptimal response. Using larger numbers of DC, MRC OX-6 had no
effect on the proliferation of the Th blasts (data not shown). Neither of the
antibodies had any effect on the response of Th blasts or memory cells to an IL-
2-containing supernatant. MRC OX-17 (anti-I-E) had no effect on the restimu-
lation of the Th blasts and Th memory cells in a secondary MLR but inhibited
the primary response by up to 30% and MRC OX-18 (anti-class I) had no effect
when added to all three assays (data not shown).

Effects of W3/25 and MRC OX-6 on Cell Interactions Between Th Cells and
DC. Th blasts and DC were preincubated with W3/25 or MRC OX-6 mAb for

| DC incubated with $5 \times 10^4$ HO Th memory cells | Percent T cells bound to DC |
|---------------------------------------------------|-----------------------------|
| $10^5$ Syngeneic (HO) DC                           | $6 \pm 2$                   |
| $2.5 \times 10^5$ syngeneic (HO) DC                | $8 \pm 2$                   |
| $10^4$ Allogeneic (HOB2) DC                        | $22 \pm 3$                  |
| $2.5 \times 10^4$ Allogeneic (HOB2) DC            | $41 \pm 4$                  |
| $10^5$ Third-party (DA) DC                         | $7 \pm 1$                   |
| $2.5 \times 10^5$ Third-party (DA) DC             | $7 \pm 2$                   |

DC and fluorescently labeled Th memory cells were mixed together and
pelleted and incubated for 15 min at 37°C. The cells were resuspended
(12 times) and the percentage of T cells bound to DC was determined
using a hemocytometer.
TABLE V

Effects of W3/25 and MRC OX-6 on Proliferation of Th Cells

| Stimulator* cells or IL-2 added | mAb* added (μg/ml) | [³H]TdR uptake (cpm X 10⁻⁵) | Unprimed Th cells (2 x 10⁴) | Th blasts (2 x 10⁴) | Th memory cells (2 x 10⁴) |
|---------------------------------|-------------------|----------------------------|----------------------------|---------------------|--------------------------|
|                                 |                   | 2.9 ± 0.5                  | 2.2 ± 0.8                  |                     |                          |
| Syngeneic DC (HO)               | W3/25 (0.2)       | 8.8 ± 1.8                  | 32.6 ± 2.9                 | 45.2 ± 4.2          |                          |
| Allogeneic DC (HOB2)            | W3/25 (2)         | 5.4 ± 1.3                  | 31.0 ± 3.0                 | 10.0 ± 2.4          |                          |
|                                 | W3/25 (20)        | 6.5 ± 1.9                  | 34.7 ± 2.4                 | 12.3 ± 2.4          |                          |
| Allogeneic DC                   | MRC OX-6 (0.2)    | 5.7 ± 1.5                  | 28.8 ± 2.7                 | 43.4 ± 5.6          |                          |
|                                 | MRC OX-6 (2)      | 5.5 ± 1.7                  | 32.6 ± 2.3                 | 15.4 ± 3.8          |                          |
|                                 | MRC OX-6 (20)     | 7.1 ± 1.3                  | 13.1 ± 2.0                 | 13.9 ± 3.2          |                          |
| IL-2                            |                   | 13.3 ± 1.3                 | 15.6 ± 2.3                 |                     |                          |
|                                 | W3/25 (20)        | —                          | 14.6 ± 2.5                 | 13.6 ± 1.9          |                          |
|                                 | MRC OX-6 (20)     | —                          | 15.9 ± 2.1                 | 16.8 ± 3.1          |                          |

* Cultures were set up as described in Table I. DC were used at 2 x 10⁴ cells per well with unprimed Th cells or Th memory cells and at 5 x 10³ cells per well with Th blasts. IL-2 was in the form of an MLR supernatant used at a 1:4 dilution.

* mAb were added at the initiation of the culture.

§ Proliferation was measured after 5 d incubation of the primary MLR and 2 d incubation when either Th blasts or Th memory cells were used.

30 min at 25°C before coculturing the cells in a clustering assay. The amount of aggregation was determined and the results in Table VI show that neither antibody had any significant effect on clustering between the T cells and DC. However, when the antibodies were included in the pelleting/resuspension assay involving Th memory cells and DC, they both inhibited the antigen-specific binding of the T cells to the DC (Table VI). Since W3/25 binds to ~50% of the DC it was important to determine whether the blocking effect of this antibody was caused by binding to the T cells or DC. Therefore, we preincubated only one of the two cell types with W3/25 mAb before the assay and found that labeling of T cells caused the inhibitory effect on the binding to DC (Table VI). In contrast pre-incubation of DC with MRC OX-6 blocks their binding to T cells. MRC OX-1 was included in this assay as a control and had no effect on the cell-cell binding.

W3/25 and MRC OX-6 Block IL-2 Production and Activation of Th Cells in the Primary MLR. Supernatants from primary MLR cultures obtained 2 d after their initiation were assayed for IL-2 activity on Con A blasts (Table VII). The inclusion of either W3/25 or MRC OX-6 at the initiation of the MLR inhibited the production of IL-2. Control experiments and previous results in Table V showed that the antibodies had no effect on the response of T blasts to IL-2; thus, they would not interfere with the assay for this factor.

In another experiment a secondary MLR supernatant containing IL-2 was included in a primary MLR between Th cells and spleen cell stimulators and the
**Table VI**

*Effect of W3/25 and MRC OX-6 on Interactions Between Th Cells and DC*

Clustering between Th blasts and DC:

| Components incubated with HO Th blasts | Percent aggregation of T cells |
|----------------------------------------|-------------------------------|
| --                                    | 6 ± 2                         |
| Syngeneic (HO) DC                     | 39 ± 4                        |
| Allogeneic (HOB2) DC                  | 44 ± 3                        |
| Allo-DC + W3/25                       | 40 ± 3                        |
| Allo-DC + MRC OX-6                    | 47 ± 4                        |

Antigen-specific binding between Th memory cells and DC:

| mAb      | Cells preincubated with mAb | Percent T cells bound |
|----------|-----------------------------|-----------------------|
| --       | --                          | 42 ± 5                |
| W3/25    | T                           | 12 ± 5                |
| W3/25    | DC                          | 39 ± 4                |
| W3/25    | T + DC                      | 14 ± 3                |
| MRC OX-6 | T                           | 38 ± 4                |
| MRC OX-6 | DC                          | 16 ± 3                |
| MRC OX-6 | T + DC                      | 18 ± 3                |
| MRC OX-1 | T                           | 37 ± 3                |
| MRC OX-1 | DC                          | 43 ± 4                |
| MRC OX-1 | T + DC                      | 40 ± 4                |

* Clustering assay was set up as described in legend to Table II. mAb were included at 20 μg/ml and the cells were preincubated in the antibodies for 30 min at 25°C before the assay.

HO Th memory cells or F1 MLNX DC were prepared and preincubated separately with mAb at 2 μg/ml at 25°C for 30 min. The cells were then washed three times. 5 × 10⁶ Th cells were then pelleted with 2.5 × 10⁵ DC and incubated for 15 min at 37°C. After resuspending the pellet, the percentage of fluorescent T cells bound to DC was determined. When syngeneic (HO) DC were incubated with the T cells, 9 ± 3% T cells were bound to DC.

The effect of W3/25 and MRC OX-6 were examined. The inclusion of the supernatant did not overcome the inhibition of proliferation induced by these mAb (Table VII). Since T blasts will proliferate in response to IL-2 in the presence of the antibodies the results suggest that the T cells are not activated to express IL-2 receptors in an MLR inhibited by either W3/25 or MRC OX-6.

**Discussion**

The results presented describe the interactions between Th cells and DC. The Th cells are used at three stages of differentiation, as unprimed resting cells, as actively dividing T blasts and as T memory cells that have reverted back to a resting state. Two types of interaction between Th cells and DC can be demonstrated. The first is an aggregation of blast cells with DC that is not dependent...
TABLE VII
Effect of W3/25 and MRC OX-6 on IL-2 Production in MLR and an Attempt to
Overcome the Inhibition of the MLR by Including MLR Supernatant

| IL-2 production in the MLR* | Proliferation $\left( {^{3}H} \text{TdT, cpm} \times 10^{-3} \right)$ | IL-2 production $\left( {^{3}H} \text{TdT, cpm} \times 10^{-3} \right)$ |
|-----------------------------|-------------------------------------------------|-------------------------------------------------|
| Responders only             | $1.9 \pm 1.1$                                    | $1.6 \pm 0.4$                                    |
| MLR                         | $78.3 \pm 4.6$                                   | $17.5 \pm 2.5$                                   |
| MLR + W3/25                 | $5.9 \pm 1.6$                                    | $2.5 \pm 1.0$                                    |
| MLR + MRC OX-6              | $3.4 \pm 1.8$                                    | $2.1 \pm 1.3$                                    |

Does the addition of an MLR supernatant overcome inhibition of MLR?*

| Proliferation                |                                               |
|------------------------------|------------------------------------------------|
| Responders alone             | $3.2 \pm 1.2$                                  |
| MLR                          | $55.1 \pm 3.8$                                 |
| MLR + IL-2                   | $142.6 \pm 7.2$                                |
| MLR + IL-2 + W3/25           | $13.2 \pm 1.9$                                 |
| MLR + IL-2 + MRC OX-6        | $10.1 \pm 2.6$                                 |

*MLR between HO W3/25* Th cells and F1 spleen cells. Antibodies were added at the initiation of the culture at 0.2 µg/ml. Proliferation was assayed after 5 d. Culture supernatants were harvested after 2 d and their IL-2 activity measured by determining their effects (at 1:4 dilution) on proliferation of 3-d Con A blasts.

The results show the crucial role of the DC in forming cell aggregates with activated T cells and also the importance of DC for the restimulation of Th blasts.

on antigenic differences between the cells. This is a rapid interaction and the aggregation could not be induced by either Mφ or B cells. The failure of B cells to induce clustering is in contrast to the results of Inaba and Steinman (28) using the mouse model, but may reflect differences in the assays used to measure clustering. Our assay measures the formation of aggregates of T cells with other cells rather than the binding of individual T cells to other cell types.

The ability to cluster with DC seems to be a property of activated T or B cells. The clustering of activated Th cells and DC in the MLR is probably of functional importance since the clusters that form during the syngeneic MLR and B cell activation contain the T cells that produce factors (e.g., IL-2 and B cell helper factor) necessary for proliferation and differentiation (3-5). However, clustering does not necessarily lead to proliferation of T cells, since it was only the allogeneic DC that induced restimulation. A trigger for proliferation must, therefore, be initiated by recognition of foreign MHC class II antigens.

The second type of interaction between Th memory cells and DC was observed in a different assay than that used to measure clustering; in this assay, the cells were brought into immediate contact, followed by vigorous resuspension. Antigen-specific binding of Th memory cells to DC was found. The shear forces applied to the cells in this assay would break weak bonds between cells. The results suggest that the antigen-specific interaction has a higher affinity than the nonspecific interaction.

The results show the crucial role of the DC in forming cell aggregates with activated T cells and also the importance of DC for the restimulation of Th blasts.
and memory cells in the MLR. Macrophages were ineffective as stimulators for T cells at any stage of the MLR, whereas B cells had some capacity to restimulate activated cells. Thus, once the Th cells have been activated there does not seem to be an absolute requirement for DC, although it is still the most effective stimulator of the MLR, consistent with other data (28). That the DC is the most potent stimulator of unprimed Th cells in the allogeneic MLR is also consistent with previous results (3, 6). Both the Th blasts and Th memory cells respond to IL-2, suggesting that, although the latter have stopped proliferating, they have not yet lost their receptors for IL-2 although we would expect this to occur ultimately.

W3/25 mAb inhibited proliferation when included in the primary MLR and blocked the production of IL-2, as previously observed (1, 15, 29). MRC OX-6 produced similar effects and both antibodies prevented the resting Th cells from becoming responsive to IL-2. Thus, in the primary MLR they must be affecting an early stage in activation.

The restimulation of Th blasts or memory cells in a secondary MLR was also inhibited by MRC OX-6 showing that these T cells were recognizing class II antigens. However, to block the proliferation of Th blasts with MRC OX-6, it was necessary to use a limiting number of DC stimulators and a high concentration of antibody. In addition, W3/25 mAb had no effect on the response of Th blasts in a secondary MLR, although it inhibited the restimulation of Th memory cells. These results suggest that there are different requirements for the restimulation of Th blasts than for the activation of primary or memory Th cells, and this is consistent with other data (28). The W3/25 antigen seems to be functionally important at times when the cells need to be triggered from a resting state.

Neither W3/25 nor MRC OX-6 had any effect on the aggregation between Th blasts and DC; however, both antibodies blocked the antigen-specific binding of Th memory cells to DC. In the latter experiments the data showed that the W3/25 mAb was acting on the T cells rather than the DC. The results suggest that the CD4 (W3/25) antigen on T cells is an adhesion molecule involved in antigen-specific cell interactions. It is therefore likely that W3/25 mAb prevents the activation of Th cells in the primary MLR by blocking their interaction with DC. MRC OX-6 could be having a similar effect by blocking recognition of the class II antigens on the DC.

Results from the work on the human CD4 (T4) and mouse L3T4 antigens (14, 30–32), which are equivalent to W3/25 antigen, are consistent with the findings in this paper. It has been suggested that these antigens are directly involved in class II recognition although this has not been proven (14, 30–32). Whether the nonspecific interactions between activated T or B cells with DC in clusters are necessary for cell activation and differentiation is unknown at present and an assessment of this requires the development of mAb to the surface molecules involved.

**Summary**

This paper describes the interactions between dendritic cells (DC) and T helper (Th) cells in the rat mixed lymphocyte reaction (MLR). Th blasts that are actively proliferating were generated in a 5 d primary MLR; resting Th memory cells were derived from a 10–12 d MLR. The DC were purified from thoracic
duct lymph derived from rats whose mesenteric lymph nodes had been removed. The results show that DC are the major stimulators in the primary MLR and also for the restimulation of Th blasts and Th memory cells. Th blasts rapidly formed large clusters when cultured with DC but not with Ia+ macrophages or B cells. This interaction was not dependent on a major histocompatibility complex (MHC) difference between the T blasts and the DC. Con A–activated T and B blasts also formed clusters when cultured with DC. Th memory cells formed small clusters with DC, but, in a different assay in which clusters are dispersed, we detected an antigen-specific interaction between Th memory cells and DC.

The monoclonal antibodies W3/25 (anti-rat CD4) and MRC OX-6 (anti-MHC class II) blocked proliferation in the primary MLR and also inhibited the restimulation of Th memory cells. However, the restimulation of Th blasts in a secondary MLR was only blocked by MRC OX-6. These results suggest that there are different requirements for the restimulation of T blasts than for the activation of primary or memory Th cells. W3/25 and MRC OX-6 did not affect the clustering of T blasts with DC but they both inhibited the antigen-specific binding of Th memory cells to DC. The data suggest that the CD4 (W3/25) antigen is involved in antigen-specific interactions between Th cells and DC.

We thank Marjorie Fowler for excellent technical assistance, Dr. Alan Williams and Dr. Don Mason for helpful criticism, Stan Buckingham for the photography, and Caroline Griffin for typing the manuscript.

Received for publication 3 May 1985 and in revised form 22 July 1985.

References
1. Mason, D. W., C. W. Pugh, and M. Webb. 1981. The rat mixed lymphocyte reaction: roles of a dendritic cell in intestinal lymph and T-cell subsets defined by monoclonal antibodies. Immunology. 44:75.
2. Czitrom, A. A., G. H. Sunshine, T. Reme, R. Ceredig, A. L. Glasebrook, A. Kelso, and H. R. MacDonald. 1983. Stimulator cell requirements for allospecific T cell subsets: specialised accessory cells are required to activate helper but not cytolytic T lymphocyte precursors. J. Immunol. 130:546.
3. Steinman, R. M., and M. C. Nussenzweig. 1980. Dendritic cells: features and functions. Immunol. Rev. 53:127.
4. Inaba, K., A. Granelli-Piperno, and R. M. Steinman. 1983. Dendritic cells induce T lymphocytes to release B cell–stimulating factors by an interleukin 2–dependent mechanism. J. Exp. Med. 158:2040.
5. Inaba, K., M. D. Witmer, and R. M. Steinman. 1984. Clustering of dendritic cells, helper T lymphocytes, and histocompatible B cells during primary antibody responses in vitro. J. Exp. Med. 160:858.
6. Klinkert, W. E. F., J. H. LaBadie, and W. E. Bowers. 1982. Accessory and stimulating properties of dendritic cells and macrophages isolated from various rat tissues. J. Exp. Med. 156:1.
7. Pugh, C. W., G. G. MacPherson, and H. W. Steer. 1983. Characterization of nonlymphoid cells derived from rat peripheral lymph. J. Exp. Med. 157:1758.
8. Williams, A. F., G. Galfré, and C. Milstein. 1977. Analysis of cell surfaces by xenogeneic myeloma-hybrid antibodies: differentiation antigens of rat lymphocytes. Cell. 12:663.
9. White, R. A. H., D. W. Mason, A. F. Williams, G. Galfrè, and C. Milstein. 1978. T-lymphocyte heterogeneity in the rat: separation of functional subpopulations using a monoclonal antibody. J. Exp. Med. 148:664.

10. Barclay, A. N. 1981. The localization of populations of lymphocytes defined by monoclonal antibodies in rat lymphoid tissues. Immunology. 42:595.

11. Jefferies, W. A., J. R. Green, and A. F. Williams. 1985. Authentic T helper CD4 (W3/25) antigen on rat peritoneal macrophages. J. Exp. Med. 162:117.

12. Thomas, M. L., and J. R. Green. 1983. Molecular nature of the W3/25 and MRC OX-8 marker antigens for rat T lymphocytes: comparisons with mouse and human antigens. Eur. J. Immunol. 13:855.

13. Bernard, A., L. Boumsell, J. Dausset, C. Milstein, and S. F. Schlossman, editors. 1984. Leucocyte Typing. Springer-Verlag, Heidelberg, Federal Republic of Germany. 45–48.

14. Dialynas, D. P., Z. S. Quan, K. A. Wall, A. Pierrès, J. Quintans, M. R. Loken, M. Pierrès, and F. W. Fitch. 1983. Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. J. Immunol. 131:2445.

15. Webb, M., D. W. Mason, and A. F. Williams. 1979. Inhibition of the mixed lymphocyte response with a monoclonal antibody specific for a rat T lymphocyte subset. Nature (Lond.). 282:841.

16. Mason, D. W., and A. F. Williams. 1980. The kinetics of antibody binding to membrane antigens in solution and at the cell surface. Biochem. J. 187:1.

17. McMaster, W. R., and A. F. Williams. 1979. Identification of Ia glycoproteins in rat thymus and purification from rat spleen. Eur. J. Immunol. 9:426.

18. Brideau, R. J., P. B. Carter, W. R. McMaster, D. W. Mason, and A. F. Williams. 1980. Two subsets of rat T lymphocytes defined with monoclonal antibodies. Eur. J. Immunol. 10:609.

19. Cantrell, D. A., R. A. Robins, C. G. Brooks, and R. W. Baldwin. 1982. Phenotype of rat natural killer cells defined by monoclonal antibodies marking rat lymphocyte subsets. Immunology. 45:97.

20. Hunt, S. V., and M. H. Fowler. 1981. A repopulation assay for B and T lymphocyte stem cells employing radiation chimaeras. Cell. Tissue Kinet. 14:445.

21. Fukumoto, T., W. R. McMaster, and A. F. Williams. 1982. Mouse monoclonal antibodies against rat major histocompatibility antigens. Two Ia antigens and expression of Ia and class I antigens in rat thymus. Eur. J. Immunol. 12:237.

22. Dallman, M. J., M. L. Thomas, and J. R. Green. 1984. MRC OX-19: a monoclonal antibody that labels rat T lymphocytes and augments in vitro proliferative responses. Eur. J. Immunol. 14:260.

23. Hsiung, L.-M., A. N. Barclay, M. R. Brandon, E. Sim, and R. R. Porter. 1982. Purification of human C3b inactivator by monoclonal-antibody affinity chromatography. Biochem. J. 203:293.

24. Spickett, G. P., M. R. Brandon, D. W. Mason, A. F. Williams, and G. R. Woollett. 1983. MRC OX-22, a monoclonal antibody that labels a new subset of T lymphocytes and reacts with the high molecular weight form of the leukocyte-common antigen. J. Exp. Med. 158:795.

25. Jefferies, W. A., M. R. Brandon, A. F. Williams, and S. V. Hunt. 1985. Analysis of lymphopoietic stem cells with a monoclonal antibody to the rat transferrin receptor. Immunology. 54:333.

26. Mason, D. W. 1981. Subsets of T cells in the rat mediating lethal graft-versus-host disease. Transplantation (Baltimore). 32:222.

27. Webb, M. 1980. Inhibition of a mixed lymphocyte reaction by a monoclonal antibody
specific for a rat T cell subset. In Proteins and Related Subjects. Vol. 28. Proteides of
the Biological Fluids. H. Peeters, editor. Pergamon Press Ltd., Oxford. 549–552.
28. Inaba, K., and R. M. Steinman. 1985. Resting and sensitized T lymphocytes exhibit
distinct stimulatory (antigen-presenting cell) requirements for growth and lympho-
kine release. J. Exp. Med. 160:1317.
29. Cantrell, D. A., R. A. Robins, and R. W. Baldwin. 1982. Rat lymphocyte subsets:
    cellular requirements for the generation of T-cell growth factor. Cell. Immunol.
    70:367.
30. Watts, T. H., A. A. Brian, J. W. Kappler, P. Marrack, and H. M. McConnell. 1984.
    Antigen presentation by supported planar membranes containing affinity purified I-
    A<sup>α</sup>. Proc. Natl. Acad. Sci. USA. 81:7564.
31. Greenstein, J. L., J. Kappler, P. Marrack, and S. F. Burakoff. 1984. The role of
    L<sub>3</sub>T4 in recognition of Ia by a cytotoxic, H-2D<sup>α</sup>-specific T cell hybridoma. J. Exp.
    Med. 159:1213.
32. Biddison, W. E., P. E. Rao, M. A. Talle, G. Goldstein, and S. Shaw. 1982. Possible
    involvement of the OKT4 molecule in T cell recognition of class II HLA antigens.
    Evidence from studies of cytotoxic T lymphocytes specific for SB antigens. J. Exp.
    Med. 156:1065.