AUTHENTIC T HELPER CD4 (W3/25) ANTIGEN ON RAT PERITONEAL MACROPHAGES

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An antigen that clearly marked T helper cells was first identified when the W3/25 mouse monoclonal antibody was raised against rat thymocytes (1, 2). The W3/25 antigen appears to be equivalent to human T4 antigen, which is now called CD4 (3), and to mouse L3T4 antigen (4–6). Henceforth, we will call the rat antigen "CD4 (W3/25)." Antibodies to CD4 antigens inhibit T helper cell responses in vitro (5, 7, 8) and the CD4 molecule has been sugested as a recognition structure for class II antigens (5, 9, 10).

Since the CD4 (W3/25) antigen seemed to have an important role in T helper cell responses (7, 8), it was a surprise when Barclay (11) showed that the W3/25 antibody labeled rat peritoneal macrophages. In humans, anti-T4 antibodies have also been shown to label macrophages (12, 13). However, thus far the molecular characteristics of the CD4 antigens from macrophages have not been established in any species. In this paper we examine this point to determine whether rat macrophages express authentic CD4 (W3/25) antigen.

Materials and Methods

Animals. BALB/c mice (H-2^d) and congenitally athymic PVG (rnu/rnu) rats were obtained from Olac (1976), Ltd. (Bicester, Great Britain). Specific pathogen–free inbred rats, PVG-RT1^c and DA-RT1^a (PVG and DA), were from the MRC Cellular Immunology Unit, Oxford.

Antibodies. Hybridomas were produced by fusing mouse spleen cells and the mouse myeloma cell lines NS1/1.Ag 4.1 (MRC OX-27 and OX-37), P3-X63/Ag 8 (MRC OX-38), or NSO/1 (MRC OX-34, -35, -36) as described (14). The antibodies used are summarized in Table I. Those not previously described are: MRC OX-27 was prepared by Dr. M. Brandon after immunization of mice with phytohemagglutinin blasts and resulted from fusion as described (20); MRC OX-34, -35, -36 were prepared by M. Puklavec, J. Green, and W. Jefferies after immunizing mice with T blasts prepared in mixed lymphocyte reactions with purified rat T helper cells against irradiated spleen (8); and MRC OX-37 and MRC OX-38 from fusions carried out by M. Puklavec and Drs. G. Woollett and M. Dallman, respectively, with mice immunized with lymphocytes or thymocyte glycoproteins as in previous studies (17, 21). Other antibodies used were purified rabbit anti–mouse IgG antibody and its $F(ab')_2$ fragment and purified $F(ab')_2$ anti–mouse Fab (22).

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TABLE I

Monoclonal Antibodies and Their Reactivity With Rat Tissue

Antibody	Specificity	Subclass	Reference
W3.25	Rat CD4 antigen	IgG1	1
MRC OX-35	Rat CD4 antigen	IgG2a	See Materials and Methods
OX-36		IgG2a	
OX-37		IgG1	
OX-38		IgG2a	
MRC OX-6	Rat MHC class II, I-A	IgG1	15
MRC OX-12	Rat kappa	IgG2a	16
W3/13	LSGP,* pan T, polymorphs, not B	IgG1	1
MRC OX-19	Pan T, rat Ly-1 equivalent	IgG1	17
MRC OX-21	Human C3b inactivator	IgG1	18
MRC OX-27	Rat MHC class I, C haplotype	IgG2a	Materials and Methods
W6/32	Human HLA, A, B, C	IgG2a	19
MRC OX-34	Pan T, other leukocytes, not B	IgG2a	Materials and Methods

* Leukocyte sialoglycoprotein.

Lymphocytes. Thoracic duct lymphocytes (TDL)[1] were collected overnight at 4°C in Dulbecco's A plus B medium (DAB) containing 20 U/ml heparin. T cells were obtained from PVG TDL after rosette depletion with MRC OX-12 antibody to remove B cells (23). Other cells were washed and prepared by standard methods (24).

Peritoneal Macrophages. PVG rats were killed by cervical dislocation under anesthetic and 15 ml of buffer containing phosphate-buffered saline (PBS), 10% DA serum, and 1 U heparin per milliliter was injected intraperitoneally into each rat and subsequently removed with a Pasteur pipette. The cells were washed twice in DAB, 5% DA serum and kept at 4°C. To enrich for macrophages the peritoneal cells ($1.5–2 \times 10^8$) were incubated for 1 h at 4°C with a mixture of tissue culture supernatants containing the monoclonal antibodies W3/13, MRC OX-6, MRC OX-34, and MRC OX-19, and 10% DA serum (see text and Table I for antibody specificities). After washing, the cells were incubated for 20 min at 4°C with sheep red blood cells coated with $F(ab')_2$ anti-mouse IgG antibody (23) and the cell suspension was layered over 2 ml of 20% (wt/vol) metrizamide (Nyegaard & Co., Oslo) in PBS, 0.2% bovine serum albumin (BSA) and centrifuged for 10 min at 600 *g* at 4°C. The nonrosetted cells were found at the interface and were collected and washed in PBS, 0.2% BSA. 25% of the starting cells were recovered and, of these, >80% were phagocytic as judged by their ability to ingest latex particles.

Labeling of Cells with Antibodies. Cells were labeled with saturating levels of monoclonal antibodies followed by fluorescein isothiocyanate (FITC)-labeled $F(ab')_2$ anti-mouse IgG antibody, and binding was analyzed on a FACS II (Becton, Dickinson & Co., Salt Lake City, UT) (2).

Biosynthetic and Surface Labeling of Cells and Immunoprecipitation. 2.7×10^7 purified peritoneal macrophages were labeled with 1.25 mCi [^{35}S]-L-methionine or [^{35}S]-L-cysteine (Amersham International Ltd., Amersham, Great Britain) for 16 h at 37°C in 5 ml of methionine- or cysteine-free Eagle's medium containing 10% dialyzed fetal calf serum (FCS). The number of cells recovered after incubation was roughly equal to those added and the viability was 60–95% in different experiments. For surface labeling with ^{125}I, 2 ×

[1] *Abbreviations used in this paper:* β_2m, β_2 microglobulin; BSA, bovine serum albumin; DAB, Dulbecco's A plus B medium; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescence isothiocyanate; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TDL, thoracic duct lymphocytes.

10^7 cells were washed in Dulbecco's modified Eagle's medium and incubated with lactoperoxidase, glucose oxidase, and 1 mCi $Na^{125}I$ (Amersham International Ltd.) as described (25).

Labeled cells were solubilized in DAB plus 1% Brij 96 or 1% Nonidet P-40; immunoprecipitations were carried out as described (6) after adding monoclonal antibody and then protein A–Sepharose 4B beads with bound rabbit anti–mouse IgG. Precipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography for [^{35}S]methionine or [^{35}S]cysteine and autoradiography for ^{125}I.

Results

New Antibodies to Rat CD4 (W3/25) Antigen and an Antibody to a Rat Pan T Antigen. The monoclonal antibodies MRC OX-35 through 38 were judged to be against the CD4 (W3/25) antigen on the basis of their pattern of cell labeling and immunoprecipitation data. On TDL all the antibodies gave the labeling pattern as shown for W3/25 and MRC OX-35 antibodies in Fig. 1*A*. In experiments to test for competitive binding it was found that, when W3/25 and MRC OX-37 or OX-38 antibodies were mixed together, the binding was indistinguishable from either antibody alone (data not shown); thus, these antibodies reacted with an identical or adjacent antigenic site. In contrast, W3/25 and MRC OX-35 or OX-36 antibodies were noncompetitive in binding, as can be seen in Fig. 1*A*, which shows additive binding when the antibodies were mixed together.

MRC OX-35 through OX-38 antibodies all precipitated bands from detergent extracts of ^{125}I-labeled thymocytes that were indistinguishable from the material immunoprecipitated with W3/25 antibody. This is illustrated by the results obtained with W3/25 and MRC OX-35 antibodies (Fig. 2, lanes *3* and *4*). That all antibodies bound to the same antigen was shown by the results obtained by passing detergent extracts from ^{125}I-labeled thymocytes through a W3/25 antibody affinity column. This removed material that could be immunoprecipitated by the noncompetitive antibody MRC OX-35 as well as by W3/25 antibody (Fig. 2, lanes *7* and *8*).

The MRC OX-34 antibody labeled more TDL than did W3/25 antibody (Fig. 1*A*) and was found to bind to all peripheral T cells and most thymocytes. This antibody was of interest because it did not label peritoneal macrophages (e.g., Fig. 1*C*) and thus could be used as a control to detect T cell contamination in experiments with antibodies against CD4 (W3/25) antigen. Although MRC OX-34 did not label peritoneal macrophages, it was not T cell specific, since many as yet undefined cells were labeled using the immunoperoxidase method on sections from rat spleen (data not shown). The thymocyte molecule detected by MRC OX-34 antibody had a molecular weight of ~52,000 (Fig. 2, lanes *2* and *6*) and was not depleted from extracts that passed through a W3/25 antibody affinity column (Fig. 2, lane *6*).

Functional effects of the new antibodies were tested by adding antibody in the form of tissue culture supernatants to in vitro cultures and stimulating lymphocyte division by allogeneic cells or concanavalin A. All the new antibodies against the CD4 (W3/25) antigen inhibited mixed lymphocyte responses by 90% at dilutions of tissue culture supernatant up to 1:90. This result is the same as previously reported for the W3/25 antibody (7, 8) and, in addition, the new

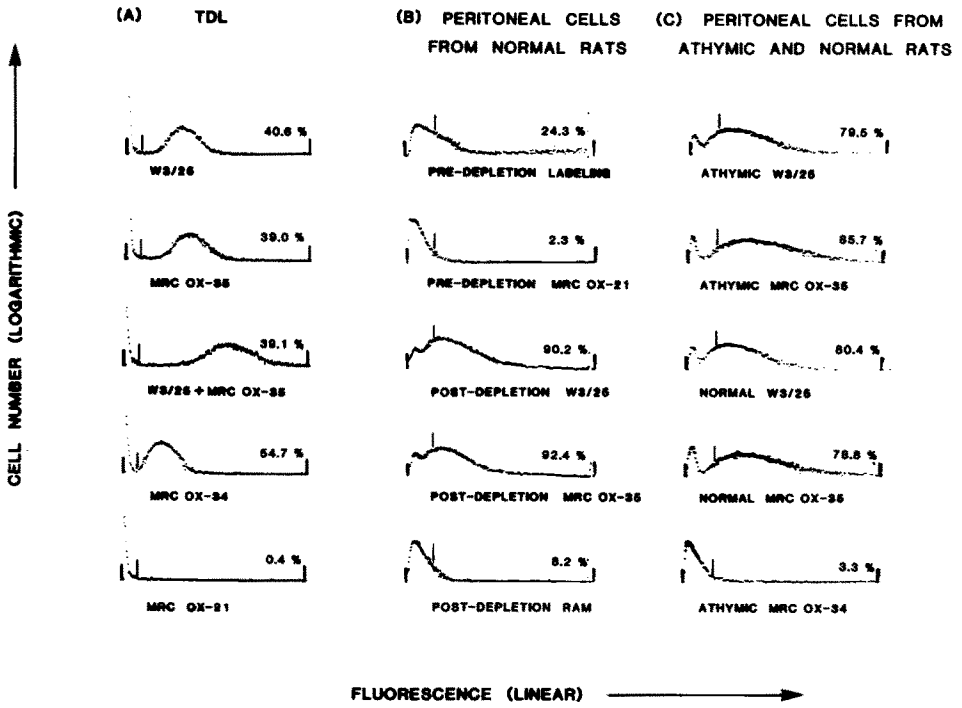

FIGURE 1. Labeling of TDL and normal and athymic peritoneal macrophages. Cells were labeled with monoclonal antibodies in the form of tissue culture supernatant followed by FITC–rabbit F(ab')$_2$ anti-mouse IgG with both steps at 4°C. Fluorescence histograms were obtained on the FACS with 10^5 cells counted for each profile. The value for the percent cells labeled with fluorescence greater than the marker is given above each profile and the antibodies are indicated below in each case. The MRC OX-21 (IgG1) antibody was used as a nonspecific control and similar control profiles were seen when the W6/32 (IgG2a) control antibody was used. The profiles are (*A*) for labeled TDL, (*B*) for macrophage-enriched populations from the peritoneum of normal rats, and (*C*) for unfractionated macrophages from normal or athymic rats. In *B*, pre-depletion labeling included addition of MRC OX-6, MRC OX-19, MRC OX-34, and W3/13 antibodies in the first step; the post-depletion RAM profile is for cells incubated with FITC–rabbit F(ab')$_2$ anti-mouse IgG only after the cells labeled with the antibody cocktail had been removed by rosette depletion. The last profile in *C* shows labeling with MRC OX-34 antibody and a similar profile was seen with MRC OX-19 antibody (1.2% labeled) and MRC OX-21 (1.3% labeled).

antibodies matched the W3/25 antibody in having no effect on proliferation stimulated by concanavalin A.

Labeling of Macrophages. Resident peritoneal cells from normal animals include ~75% macrophages of which ~10% are activated as assessed by expression of Ia antigen (11). In our experiments the following unwanted cells were removed from the peritoneal cell population by rosette depletion after labeling with appropriate antibodies (see Table I): B cells and Ia^+ macrophages (MRC OX-6 antibody), T cells (MRC OX-19, OX-34, and W3/13 antibodies), and granulocytes (W3/13 antibody). The labeling of peritoneal cells with the above antibody cocktail before and after depletion is shown in Fig. 1*B* in the first and last profiles. >80% of the recovered cells were able to ingest latex beads and >90% were labeled after incubation with any of the antibodies against CD4 (W3/25)

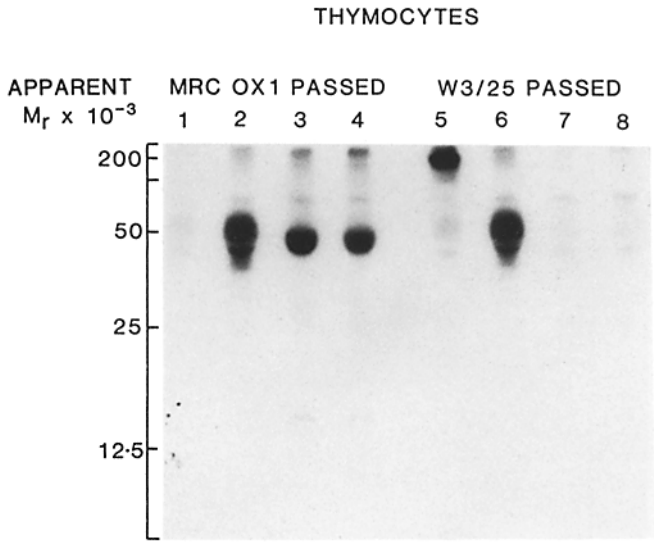

FIGURE 2. 12% SDS-PAGE of ^{125}I-labeled thymocyte glycoproteins passed through MRC OX-1 or W3/25 affinity columns. 10^8 thymocytes were labeled with Na^{125}I by the lactoperoxidase, glucose oxidase method. The membrane proteins were solubilized in 4 ml 1% Brij 96 and, after centrifugation, 1.9 ml of extract was passed through either MRC OX-1 or W3/25 affinity columns (5 ml of 10 mg IgG per milliliter Sepharose 4B) with the MRC OX-1 column being used as a control. Immunoprecipitation was carried out using the material passing through the columns with the monoclonal antibodies: MRC OX-1 (lanes *1* and *5*), MRC OX-34 (*2* and *6*), W3/25 (*3* and *7*), MRC OX-35 (*4* and *8*). All samples were reduced with 5% 2-mercaptoethanol.

antigen, as shown for W3/25 and MRC OX-35 antibodies (Fig. 1*B*). The labeling could not be due to Fc receptors since incubation with control antibodies of subclass IgG1 (MRC OX-21) (Fig. 1*B*) or IgG2a (W6/32) (not shown) labeled only a small percentage of the cells.

Peritoneal cells from athymic rats were studied to test whether the presence of CD4 (W3/25) antigen on macrophages depended upon T cells. The cells from athymic rats were used without macrophage enrichment procedures. It can be seen in Fig. 1*C* that W3/25 and MRC OX-35 antibodies each label ~75% of the peritoneal cells and that the labeling was similar to that seen with cells from normal rats (Fig. 1*C*). The MRC OX-19 and OX-34 antibodies that label T cells, however, labeled only 2% more athymic peritoneal cells than a control antibody (see legend to Fig. 1*C*).

Molecular Forms of CD4 (W3/25) Antigen on Macrophages. T cells and macrophages purified with rosetting techniques were surface labeled with ^{125}I and the CD4 (W3/25) antigen was immunoprecipitated from detergent extracts prepared from the cells. Fig. 3 shows that a molecule with the same molecular form was precipitated from both cell types. From T cells, W3/25 antibody precipitated a band at 46,000 mol wt in the unreduced form (Fig. 3, lane *2*) and at 53,000 mol wt in the reduced form (Fig. 3, lane *8*). The shift in apparent molecular weight upon reduction can be clearly seen in comparison with the band precipitated by MRC OX-34 antibody (Fig. 3, lanes *3* and *9*), which has a molecular weight of ~52,000 regardless of reduction. From macrophages, the W3/25 antibody

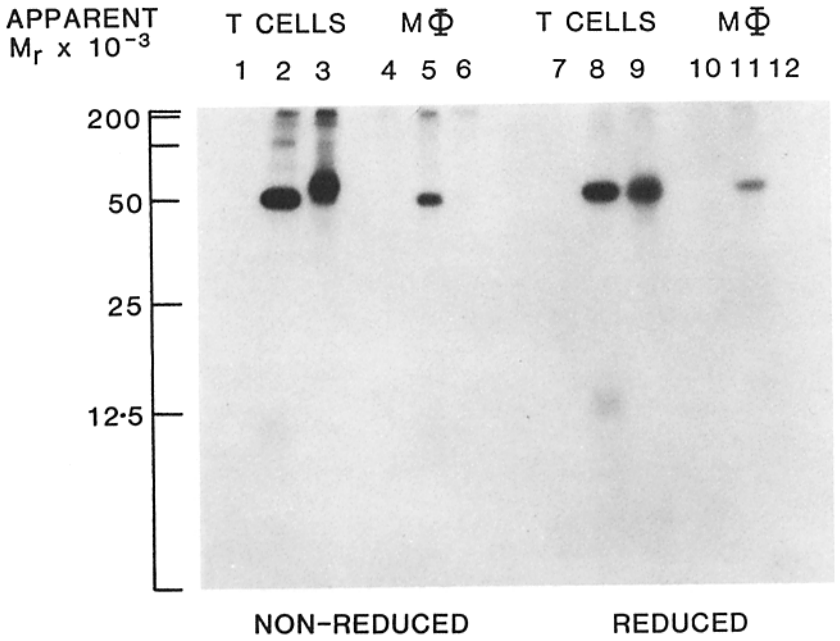

FIGURE 3. 15% SDS-PAGE of ^{125}I-labeled purified T cells and peritoneal macrophages ($M\phi$). After solubilization in 1% Nonidet P-40, immunoprecipitation was carried out with control antibody MRC OX-21 (*1, 4, 7,* and *10*), W3/25 (*2, 5, 8,* and *11*), or MRC OX-34 (*3, 6, 9,* and *12*). Lanes *1–6* were run nonreduced and *7–12*, reduced, with 5% 2-mercaptoethanol.

precipitated a band with identical mobility to the T cell product in the unreduced and reduced state (Fig. 3, lanes *5* and *11*). No band was precipitated from macrophages with the MRC OX-34 antibody (Fig. 3, lanes *6* and *12*) or MRC OX-19 antibody (data not shown) and this confirms the absence of contaminating T cells in the macrophage preparation.

The CD4 (W3/25) antigen was also immunoprecipitated from peritoneal cells of athymic rats and again a band of the predicted size was found (Fig. 4, lanes *2* and *4*). In this case the cells were used without depletion but this does not affect interpretation of the results since significant numbers of T cells could not be detected amongst the peritoneal cells of athymic rats (Fig. 1 *C*, last profile).

In an attempt to prove endogenous synthesis of CD4 (W3/25) antigen by macrophages, we labeled purified preparations of normal peritoneal macrophages with [^{35}S]cysteine or [^{35}S]methionine. Despite background problems in some experiments, a specific band was consistently seen at the molecular weight for CD4 (W3/25) antigen; one result for unreduced material is shown in Fig. 5, in which lane *2* shows material precipitated with W3/25 antibody. However, this band, which was observed in six experiments, was always at low intensity, as seen by comparing it with material precipitated with MRC OX-27 antibody, which reacts with rat class I antigen (Fig. 5, lane *3*). In contrast, the CD4 (W3/25) antigen on T cells was quite well labeled in comparison with class I antigen (data not shown). An additional problem in metabolic labeling experiments was the fact that the MRC OX-34 antigen did not provide an effective control for T cell contamination because of low incorporation of either [^{35}S]cysteine or [^{35}S]-

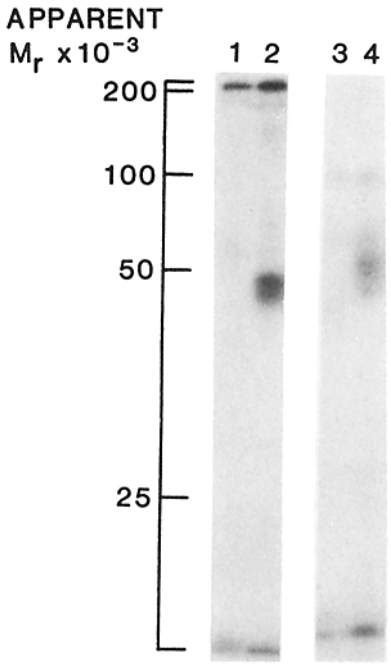

FIGURE 4. 10% SDS-PAGE of ^{125}I-labeled peritoneal macrophages from nude rats. After solubilization in 1% Nonidet P-40 immunoprecipitation was with control antibody MRC OX-21 (*1* and *3*) and W3/25 antibody (*2* and *4*). Lanes *1* and *2* are nonreduced and *3* and *4*, reduced, using 5% 2-mercaptoethanol.

methionine into this band. Thus, the metabolic labeling experiments did not provide conclusive evidence for endogenous synthesis of CD4 (W3/25) antigen by macrophages.

Finally, one previously unreported result concerning the molecular form of CD4 (W3/25) antigen was seen after surface labeling with ^{125}I material. This was the presence of an apparently specific band at 11,500 mol wt, unreduced, and 13,000, reduced, that was precipitated by antibodies against the CD4 (W3/25) antigen. This band was seen in immunoprecipitation from T cells (Fig. 3, lanes *2* and *8*) and macrophages (Fig. 3, lanes *5* and *11*; poorly shown in this photograph but visible in the radiographs). The band was not β_2-microglobulin (β_2m), since it did not coincide with the β_2m band from rat class I antigen when compared with the CD4 (W3/25) antigen on the same gels (data not shown). Also, the low molecular weight band could not be convincingly detected in metabolic labeling experiments using T cells or macrophages. Furthermore, in structural studies on the rat CD4 (W3/25) antigen (W. Jefferies, unpublished results), the low molecular weight band was not detected as a band labeled by silver staining in purified preparations of the antigen. At this stage no clear conclusion can be made concerning the low molecular weight band.

Discussion

The results presented establish that antibodies against the CD4 (W3/25) antigen label a molecule on peritoneal rat macrophages that is indistinguishable

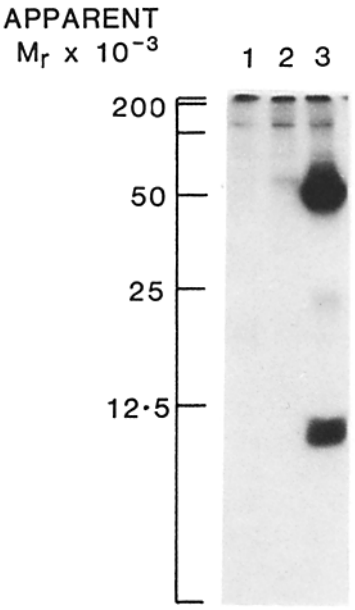

FIGURE 5. 15% SDS-PAGE of [^{35}S]cysteine-labeled purified peritoneal macrophages. After labeling, cells were solubilized in 1% Nonidet P-40 and immunoprecipitated with MRC OX-21 (*1*), W3/25 (*2*), and MRC OX-27 (track *3*). All samples were run nonreduced.

from that on T cells. This conclusion is reached because antibodies against two distinct epitopes on the CD4 (W3/25) antigen label macrophages from normal and athymic animals and because the antigen immunoprecipitated from detergent extracts of ^{125}I-labeled macrophages is indistinguishable from the T cell antigen.

A second question is whether or not the macrophage CD4 (W3/25) antigen is an endogenous product or might be acquired from T cells. There is strong evidence for synthesis by macrophages from studies on athymic peritoneal cells that were labeled by W3/25 antibody in the same way as peritoneal cells from normal rats, even though athymic animals have few if any functional helper T cells (26). Attempts to show endogenous synthesis by biosynthetic labeling were only partially successful, in that weak labeling of the CD4 (W3/25) antigen band occurred when macrophages were incubated with [^{35}S]cysteine or [^{35}S]methionine. The CD4 (W3/25) antigen is a minor antigen on T cells and macrophages, both of which bind ~25,000 molecules of W3/25 antibody per cell (27 and W. Jefferies, unpublished results). The poor biosynthesis by macrophages was not due to a high percentage of the cells dying but could be due to low turnover of the antigen or to a change in biosynthesis in culture. Another case where biosynthesis does not correlate with surface antigen expression is that of mouse thymocytes, where major histocompatibility complex (MHC) antigens are major biosynthetic products even though they are expressed on only ~15% of the cells (28). Thus, in spite of weak biosynthetic labeling, we believe that the athymic rat data provide a strong case for endogenous synthesis, in accord with data on expression of human CD4 antigen. In a study by Moscicki et al. (13), the molecular form of human macrophage CD4 antigen was not established but

antibodies against distinct epitopes of CD4 labeled the cell line that was considered to be of macrophage origin. In this case, the antigen could not have been acquired from T cells.

Rat macrophages from a variety of tissues are labeled by W3/25 antibody. Initially, labeling of peritoneal, spleen, and lymph node macrophages was reported (11); more recently (29), Kupffer cells in rat liver have been shown to be W3/25 positive. Also, cells with a dendritic morphology in sections of liver, heart, and kidney were labeled with W3/25 antibody (29) and may be macrophages or dendritic cells of the type described by Steinman and Nussenzweig (30). At least 50% of monocytes from rat blood have also been shown to be labeled by W3/25 antibody (A. Robinson, personal communication). In humans, CD4 antigen has been detected on macrophages in sections of intestine and on blood monocytes (12, 13).

The CD4 antigen of T helper cells is clearly implicated in the function of these cells in rat, man, and mouse, since anti-CD4 antibodies profoundly inhibit T cell responses to antigen. Furthermore, it has been established (7, 8) that this inhibition works at the level of T cells rather than accessory cells. It has been suggested that the CD4 molecule functions to recognize class II MHC antigen on antigen-presenting cells but there is conflicting evidence (5, 9, 10, 31). Speculation on the function of the CD4 antigen has not taken into account the fact that it is expressed on macrophages and possibly other accessory cells. It remains to be established whether the CD4 molecule can be associated with any known macrophage function.

Summary

The rat W3/25 antigen that appears to be equivalent to human CD4 (T4) antigen is expressed on thymocytes and T helper cells and plays a role in the response of T helper cells to antigen. The W3/25 and anti-T4 antibodies also label macrophages. In this paper we examine whether the macrophage antigen is the same as that on T cells. New monoclonal antibodies against the rat CD4 antigen, MRC OX-35 through OX-38, are described, all of which label peritoneal macrophages from normal and athymic rats. The molecular weight of W3/25 antigen on macrophages is indistinguishable from that on T cells. We conclude that macrophages express authentic CD4 (W3/25) antigen.

Another new monoclonal antibody, MRC OX-34, labels an antigen of 50–54,000 mol wt that is expressed on rat T but not B cells or peritoneal macrophages. It was used to control for the presence of any T cell products in immunoprecipitation from rat macrophage extracts.

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