ACTIVATION OF RAT T LYMPHOCYTES BY ANTI-CD2 MONOCLONAL ANTIBODIES

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The CD2 antigen (T11, E-rosette receptor) was first identified on human T lymphocytes with mAbs (1-3). The antigen is a glycoprotein of 50,000 apparent $M_r$ and recently the protein sequences for CD2 of human (4, 5), rat (6), and mouse (7) have been derived from cDNA sequences. In each case the sequence indicates two external domains that are Ig related, one transmembrane sequence, and a cytoplasmic domain with 115-116 amino acids.

In humans, anti-CD2 mAbs were initially found to inhibit T lymphocyte proliferation stimulated by mitogens, soluble antigens, or allogeneic cells (3, 8-10). Then particular combinations of antibodies were found to be mitogenic and some single antibodies also had this property when present with phorbol esters (11-13). CD2 is expressed in human thymocytes from an early stage and triggering of cell division by a natural ligand of CD2 has been suggested as an alternative route for cell activation to the T lymphocyte antigen receptor (11, 14). There is strong evidence that LFA-3 (alternatively identified as sheep erythrocyte TUTS) is the natural ligand for CD2 (15-17) and that the interaction between CD2 and LFA-3 can mediate adhesion of lymphoid cells. Also, purified LFA-3 can synergize with anti-CD2 to stimulate mitogenesis (18).

In the rat, the MRC OX-34 antibody is specific for CD2 and labels most T lymphocytes and thymocytes (6, 19). However, OX-34 also labels rat splenic macrophages, while similar cells in humans have not been reported to be CD2+. The MRC OX-34 mAb had no clear-cut effect on the function of the T lymphocytes, but has been recently shown to inhibit the cytolytic function of an NK-like cell line (20).

In the present work we have raised new mAbs against rat CD2 and show that one pairing of these activates DNA synthesis in T lymphocytes. The response was dependent on accessory cells and was potentiated by an anti-CD5 mAb and inhibited by anti-IL-2-R antibody.

Materials and Methods

Animals. Inbred PVG-RT1+ and PVG-RT1-+ rats (referred to as HO and HO.B2, respectively) were from the Specific Pathogen Free Unit of the MRC Cellular Immunology Unit. BALB/c mice were from the Sir William Dunn School of Pathology.

Monoclonal and Other Antibodies. Cloned hybrid cell lines that produce the mAbs MRC OX-53, OX-54, and OX-55 were from two fusions between mouse spleen cells and the mouse myeloma line NSO/I using standard procedures (21). The spleen cells were from BALB/c mice immunized with purified rat CD2 (6): two 7 µg i.m. injections of CD2 emulsified in CFA
given on days 0 and 14, followed by one 7 μg i.p. injection of potassium alum precipitate of the pure CD2. Mice were boosted with either 5 × 10^7 washed HO thymocytes given intravenously on day 56 (MRC OX-53) or with 15 μg of the pure protein given intraperitoneally on day 154 (OX-54 and OX-55). MRC OX-53, OX-54, and OX-55 are all of the IgG1 isotype.

Other mAbs used (referenced below or in reference 19) were: W3/13, IgG1, anti-rat leukosialin (LSGP); W3/25, IgG1, anti-rat CD4; MRC OX-1, IgG1 and MRC OX-30, IgG2a, against distinct epitopes of the leucocyte common antigen (L-CA); MRC OX-22, IgG2a, anti-L-CA, restricted determinant; MRC OX-6, IgG1, anti-MHC class II (non-polymorphic); MRC OX-12, IgG2a, anti-rat κ chain; MRC OX-19, IgGI, anti-rat CD5 (Ly-1); MRC OX-34, IgG2a, anti-rat CD2; MRC OX-39, IgG1 and NDS 62 (Tellides, G., M.J. Dallman, P.J. Morris, manuscript in preparation), IgG1, both anti-IL-2-R mAbs; MRC OX-21, IgG1, anti-human C3b inactivator, used as a control mAb. Antibodies were used as tissue culture supernatants or purified IgG and F(ab')2 fragments and labeled with fluorescein (RAM-FITC) or 125I (125I-RAM) as described.

Other rabbit anti-mouse IgG antibodies (RAM) and their F(ab')2 fragments were prepared and labeled with fluorescein (RAM-FITC) or 125I (125I-RAM) as described.

Cells. Cervical lymph nodes, thymuses, and spleens were removed aseptically and teased with watchmakers' forceps in ice-cold Dulbecco's A + B medium (DAB) containing 0.2% BSA. The cells were filtered through lens tissue, washed twice in DAB/BSA, and resuspended in RPMI-1640 medium containing 2.5 × 10^-5 M 2-ME, antibiotics, and 5% (vol/vol) FCS (Gibco Ltd., Paisley, Scotland) (RPMI/5% FCS).

Blasts. Either HO thymocytes, lymph node cells (LNC), or spleen cells were incubated at 2–5 × 10^6 cells/ml in RPMI/5% FCS for 72 h at 37°C in a humidified 5% CO2 incubator with Con A, used at a final concentration of 5 μg/ml. Cells were harvested, washed in RPMI/5% FCS, and the blasts were separated on an Isopaque-Ficoll gradient (Pharmacia GB Ltd., Middlesex, U.K.) and centrifuged at 750 g for 20 min at room temperature. Cells recovered from the interface were washed twice in RPMI/5% FCS.

Tissue Culture. All cultures were in 0.2 ml in U-bottomed wells of 96-well microtiter plates at 37°C with 5% CO2. Medium was RPMI-1640 plus 5% FCS. Stimulator cells were spleen cells γ-irradiated with a 106 Cs source (Gamma Cell 40; Atomic Energy of Canada Ltd., Kanata, Ontario, Canada) at 0.85 Gy/min to yield a dose of 20 Gy.

Proliferation Assays. For alloantigen-induced proliferation, a primary MLR was set up by culturing together 2.5 × 10^5 HO LNC plus 5 × 10^5 HO.B2 stimulator cells for a total of 90 h. For proliferation induced by mAbs 2.5 × 10^5 HO LNC were cultured with or without 5 × 10^5 HO stimulator cells for a total of either 72 or 90 h. mAbs were added at the beginning of the culture period and were used as tissue culture supernatant unless otherwise stated. PMA at 2 ng/ml and IgG RAM or F(ab')2 RAM at 20 gg/ml were also added at the beginning of culture when used. Cultures were pulsed with 0.5 μCi tritiated thymidine (Amersham International, Amersham, U.K.) 18 h before harvesting onto glass fiber filters (Whatman Ltd., Maidstone, U.K.). Results are expressed as arithmetic means of quadruplicate cultures ± SD.

Generation of CTL and 51Cr-release Assay. Cytotoxic cells were generated in an MLR as above cultured for 5 d. The Y3 rat myeloma cell line carrying the RT1^I MHC antigens (21) was used as a control to assay the CTL activity. The NSO mouse myeloma cell line (21) was used as a control target for non-specific killing. Percent 51Cr release was calculated using the formula: percent specific release = 100 × ([experimental release – spontaneous release]/[maximum release – spontaneous release]).

Nonspecific Killing. 51Cr release was used to measure the effect of mAbs on nonspecific killing (26). Effector cells were incubated in RPMI/2.5% FCS containing 2 mg/ml carbonyl iron for 1 h at 37°C in 5% CO2. Phagocytic cells were then removed magnetically. Remaining cells were washed twice in DAB/BSA, resuspended in RPMI/2.5% FCS, and twofold serial dilutions were made. The NSO mouse myeloma cell line was prelabeled with 51Cr and used as the target at 10^4 cells per well with serial dilutions of effector cells. 51Cr release

1 Abbreviations used in this paper: Cas, supernatant from Con A-activated rat spleen cells; L-CA, leucocyte common antigen; LNC, lymph node cells; RAM, rabbit anti-mouse IgG antibodies.
was measured after a 4-h incubation of effectors and targets. The percentage specific 51Cr release was calculated as above.

**Immunoprecipitation.** HO thymocytes were 125I surface labeled using the lactoperoxidase/glucose oxidase method. Cell membranes were solubilized in 1% NP-40 and antigens immunoprecipitated and analyzed as described (27).

**Indirect Binding Assays and Antibody Blocking Assays.** These were at 4°C unless otherwise stated and 5 x 10⁶ cells were incubated with mAbs and 125I-F(ab')² RAM in successive 60-min incubations with two washes using DAB/0.25% BSA after each step. The system was calibrated for molecules of first antibody bound by including labeling of thymocytes with W3/13 mAb and assuming that 38,000 molecules were bound (25, 28). Competition in indirect binding assays was assessed by seeing whether binding was additive when antibodies were mixed (28).

To directly assess competition between antibodies an 125I-labeled mAb was mixed with an unlabeled mAb and then the mixture was incubated with 5 x 10⁶ thymocytes for 2 h. Cells were washed and counted as before (25).

**Other Methods.** Labeling for FACS II (Becton Dickinson and Co., Mountain View, CA) analysis (28); immunoperoxidase labeling on cryostat sections (29). Tissue culture supernatant containing IL-2 was obtained as a lectin-free supernatant (Cas) from Con A-activated rat spleen cells (30). This was nonmitogenic when used alone.

**Results**

**The Nature of the Antigen Recognized by MRC OX-53, OX-54, and OX-55 mAbs.** In preliminary studies, the new mAbs were shown to have a tissue distribution indistinguishable from that of MRC OX-34. In particular splenic tissue macrophages were labeled (not shown) as well as most thymocytes and T lymphocytes. Labeling profiles for the mAbs on lymph node cells and activated T lymphocytes are shown in Fig. 1, a and b.

To establish that the mAbs were against CD2 sequential immunoprecipitations were carried out in which solubilized 125I-labeled cell surface molecules were precleared with OX-34 mAb before immunoprecipitation with the other mAbs. All the mAbs immunoprecipitated similar bands of 50 x 10³ apparent M₀ and OX-34 removed the material that could react with the OX-53, 54, and 55 mAbs but not the CD4 antigen that binds to the W3/25 mAb (Fig 1 c). Furthermore, when L cells were transfected with rat CD2 cDNA, the transfectants but not the normal L cells were labeled with MRC OX-34, OX-53, OX-54, and OX-55 mAbs (He Qi; Barclay, A. N. , and A. F. Williams, unpublished data). Thus, there is no doubt that the four antibodies all bind to CD2.

**Site Numbers for mAb Binding.** Table I shows the molecules per cell for each anti-CD2 mAb binding at saturation to resting and blast cells derived from the thymus or lymph node. The antibodies bound in roughly similar amounts with differences that might be accounted for by differences in the proportion of bivalent/monovalent binding. Although saturating binding was similar the level of antibody needed for saturation was 10-fold greater for OX-54 and OX-55 antibodies than for OX-34 or OX-53. Such a difference is unusual and whether or not this might be due to differences in kinetics of binding remains to be established.

Between T blasts and resting cells, the increment in binding was about threefold. The volume of T blasts is about three to five times greater than resting T cells, and thus there was no increase in antigen density detected between resting and activated cells.

**Competition Between mAbs.** Initially, competition was assessed using saturating in-
direct binding assays with mixtures of mAbs added in the first step. In other studies, mAbs against separate molecules or against nonoverlapping epitopes on the same molecule have given additive binding when mixed in the indirect binding assay (28). An example of this is shown for noncompeting mAbs against L-CA (OX-1 and OX-30) in Table II. In the case of the anti-CD2 mAbs, OX-34 and OX-53 are concluded to be fully competitive since they showed no additive binding at all. With combina-
TABLE I

Quantitation of mAb Binding to Thymocytes and T Blasts at Saturation

| mAbs   | Thymocytes | Thymic blasts | LN T cells | LN T blasts |
|--------|------------|---------------|------------|-------------|
| OX-34  | 1.1 x 10^4 | 3.6 x 10^6    | 1.4 x 10^4 | 4.7 x 10^4  |
| OX-53  | 7.0 x 10^3 | 2.5 x 10^4    | 9.0 x 10^3 | 2.9 x 10^4  |
| OX-54  | 1.4 x 10^4 | 1.8 x 10^4    | 1.4 x 10^4 | 2.1 x 10^4  |
| OX-55  | 6.0 x 10^3 | 2.3 x 10^4    | 5.0 x 10^3 | 3.3 x 10^4  |

5 x 10^6 cells were incubated for 2 h with saturating levels of mAbs, washed, and then incubated with 0.6 μg 125I-F(ab')2 RAM (5 x 10^5 cpm) per assay. Specific binding was determined and from this molecules of mAb bound were calculated via calibration of the assay with W3/13 mAb, which is known to bind at a level of 38,000 molecules per rat thymocyte (25, 28). Similar results were obtained in two other experiments.

Additive Binding to HO Thymocytes with Mixtures of mAbs under Saturating Conditions

| Second mAb | First mAb | OX-34 | OX-53 | OX-54 | OX-55 |
|------------|-----------|-------|-------|-------|-------|
| None       | OX-34     | 24,107 ± 1,236 | 25,569 ± 2,472 | 23,723 ± 1,713 | 23,875 ± 823 |
|            | OX-34     | –     | 25,052 ± 854 | 33,004 ± 3,202 | 31,768 ± 2,348 |
|            | OX-54     | –     | –     | 36,195 ± 2,114 | –     |
|            | OX-34 + OX-54 | –   | –     | –     | 41,586 ± 2,475 |
| Control    | OX-1      | 114,316 ± 7,642 | 86,478 ± 3,335 | –     | –     |
|            | OX-1      | –     | 182,338 ± 13,093 | –     | –     |

5 x 10^6 washed thymocytes were incubated with 10 μl of each mAb used as purified IgG. Saturating concentrations for each mAb had been previously determined. After three washes the cells were incubated with 0.6 μg 125I-F(ab')2 RAM (10^6 cpm) per assay for 1 h. The cells were washed and cell-bound radioactivity was determined.
posed on a target antigen the level of second antibody that can bind may be less per molecule than with each antibody added alone.

*The Effect of MRC OX-54 and OX-55 on Rat Lymphoid Cell Proliferation.* None of the anti-CD2 mAbs used alone, either in the presence or absence of Cas, were able to stimulate DNA synthesis in HO LNC or thymocytes (Fig. 3, a and b). However, if MRC OX-54 and OX-55 were used together in the presence of Cas, low but significant levels of thymidine incorporation were seen with the LNC (Fig. 3 a). In some experiments the two antibodies with no additional Cas were sufficient to produce some incorporation, but this result was variable. Thymocytes did not respond to the two mAbs even in the presence of Cas.

Some variation in results might have been due to variable levels of accessory cells and Fig. 3 c and d, show that addition of $5 \times 10^5$ irradiated syngeneic spleen cells
FIGURE 3. Effect of accessory cells on proliferative response of LNC and thymocytes to anti-CD2 mAbs. HO LNC or thymocytes (2.5 x 10^5) were incubated either without (a, b) or with (c, d) 5 x 10^5 irradiated syngeneic spleen cells for 72 h in RPMI/5% FCS. mAbs were added at the beginning of culture as tissue culture supernatant. Cells were cultured in the absence (solid columns) or presence (cross hatched columns) of 10% Cas.

considerably stimulated the response of both LNC and thymocytes. The anti-CD2 mAbs used singly still gave no proliferation but the combination of MRC OX-54 and OX-55 caused proliferation in both cell types that was not significantly enhanced by the addition of Cas. No other combination of anti-CD2 mAbs gave stimulation even in the presence of accessory cells.

The effect of other mAbs on stimulation of cells by OX-54 and OX-55 was examined and the results were contrasted with the effects of the same antibodies on the MLR. OX-19 (anti-CD5) is known to stimulate the MLR (23) and this also stimulated the C132-mediated proliferation (Fig. 4 a). The quantitative effect was reproducibly greater for the CD2 proliferation system than for the MLR.

mAbs against class II MHC, CD4, and the IL-2-R are all known to inhibit the MLR as shown in Fig. 4 d. In the CD2 system, the anti-class II mAb was ineffective while the anti-CD4 antibody was inhibitory, but not in the form of F(ab')2, which does however inhibit the MLR. The anti-IL-2-R antibody also inhibited the CD2-mediated proliferation.

In studies on human cells anti-CD2 mAbs can inhibit the MLR, but this is not reproducibly seen in the rat. The result shown in Fig. 4 d is one where inhibition with the OX-34 anti-CD2 mAb was seen. In contrast to variable inhibition in the MLR the OX-34 and OX-53 mAbs reproducibly gave strong inhibition of proliferation induced by OX-54 and OX-55. This occurred despite the fact that OX-34 did not inhibit the binding of OX-54 or OX-55 (Fig. 2) and that competition between OX-54 and OX-55 was not effected by binding of OX-34 at the levels used in the functional assays (data not shown).
Factors affecting LNC proliferation. (a) $2.5 \times 10^5$ HO LNC induced to proliferate by mAbs MRC OX-54+OX-55 in the form of tissue culture supernatant, in the presence of $5 \times 10^5$ irradiated HO spleen cells. (b) $2.5 \times 10^5$ HO LNC induced to proliferate by $5 \times 10^5$ HO.B2 irradiated spleen cells in an allogeneic MLR. Additional mAbs were added to the cells at the beginning of incubation and the effects of these on proliferation studied by measuring $[^3H]$thymidine incorporation. Additional mAbs were used in the form of either tissue culture supernatant or purified IgG or F(ab')2 at 20 μg/ml. Incubation was for 72 h in RPMI/5% FCS (a, c) and for 90 h (b, d).

In studies on human T cells there are some cases where DNA synthesis is stimulated by one anti-CD2 antibody plus phorbol ester (11, 12). Using PMA at a concentration of 2 ng/ml it was found that none of the anti-rat CD2 mAbs used singly would give proliferation with PMA (Fig. 5, a and b). Higher levels of PMA were toxic for rat T cells. Furthermore, PMA did not augment the proliferation induced by MRC OX-54 and OX-55 used together. The effects of crosslinking the single mAbs with either RAM IgG or RAM F(ab')2 were also investigated. No single mAbs were found to be mitogenic with either crosslinker, even in the presence of PMA. However, although proliferation induced by MRC OX-54 and OX-55 was not affected by the addition of RAM IgG, the addition of RAM F(ab')2 prevented almost all $[^3H]$thymidine incorporation. These results were unaffected by the presence or absence of PMA. The inhibition with F(ab')2 RAM may occur because interaction with Fc receptors on accessory cells is essential for the response to occur. F(ab')2 RAM may obscure the Fc of the mAbs without providing its own Fc for interaction, as would be the case with the use of IgG RAM.

Effect of Anti-CD2 mAbs on Natural Killing and CTL-mediated Killing. None of the anti-CD2 mAbs either singly or in combination had any effect on nonspecific killing when the NSO mouse myeloma cell line was used as a target for HO spleen cells. This was also true for the specific CTL-mediated killing of Y3 myeloma cells by CTL generated in a primary MLR (data not shown).

Discussion

Stimulation of mitogenesis in T lymphocytes can be initiated by a variety of mAbs reacting with molecules not known to be associated with the TCR complex. The target antigens include Thy-1 and LY-6 in the mouse (31, 32) and CD2 (12, 13), leukosialin (sialophorin) (33), and CD28 (T44) (34) in humans. The results herein show
that CD2 can also be a mitogenic target for rat T lymphocytes. The requirements were that the OX-54 and OX-55 mAbs were present along with an undefined population of accessory cells. It seemed likely that the accessory cells were required for more than the crosslinking of the mAbs, since crosslinking with F(ab')2 RAM inhibited the response possibly by masking Fc determinants (see Results). Phorbol ester did not potentiate the response or allow stimulation via one mAb.

The MRC OX-54 and OX-55 mAbs showed a curious pattern of competitive binding, in that OX-54 could substantially block OX-55 binding but the reverse situation did not occur. As both antibodies were effective in immunoprecipitation it seemed unlikely that this was due to differences in affinity, and OX-55 did not block OX-54 binding even if cells were preincubated with the OX-55 mAb (data not shown). This phenomenon is similar to that seen in crossblocking between the anti-human CD2 mAbs GT2 and anti-D66 (35), which also synergise to stimulate DNA synthesis. The OX-34 and OX-53 mAbs did not show any blocking of OX-54 and OX-55 binding, yet these mAbs inhibited the stimulatory effects of OX-54 and OX-55. This is a curious result given that crosslinking of OX-34 on an NK-like cell line can trigger release of Ca2+ and turnover of inositol phosphates (20).

The rat CD2 triggering data seems quite similar to the human work with the exception that: (a) all the rat CD2 determinants so far described are expressed on resting and activated cells whereas in humans the CD2 T113 epitope is thought to be specific to activated cells (11); (b) synergism of one CD2 mAb and phorbol ester to trigger the cells is not seen in the rat; (c) accessory cells are thus far essential for triggering the rat cells; (d) the anti-CD2 mAbs are not inhibitors of specific or nonspecific cytotoxic cells, although the OX-34 mAb sometimes shows inhibition of the MLR and has been shown to block killing by an NK-like cell line (20).

In humans, CD2 has been suggested as a target for stimulation of T cells by a
pathway that is independent of TCR triggering with a particular role in thymopoiesis (14). DNA synthesis can be triggered in rat thymocytes by the anti-CD2 mAbs, but it remains to be seen whether the activity is due only to thymocytes of immunocompetent phenotype.

Summary

Rat T cells and thymocytes were induced to proliferate by a pair of mAbs, MRC OX-54 and MRC OX-55, directed against rat CD2. Accessory cells were required but their role was not simply for crosslinking of the two mAbs, as neither MRC OX-54 nor MRC OX-55 alone, in the presence of a crosslinking second antibody, caused T cell mitogenesis. Nor could the phorbol ester PMA replace either antibody. The two mAbs recognized distinct epitopes on rat CD2; however, MRC OX-54 could partially block MRC OX-55 binding whereas the reverse situation was not seen. A further CD2 epitope was recognized by two mutually competitive mAbs, MRC OX-34 and MRC OX-53, which were not mitogenic. Neither MRC OX-34 nor MRC OX-53 affected the binding of MRC OX-54 or MRC OX-55, yet they prevented the mitogenic effect induced by these mAbs. The presence of mAbs against CD4 and the IL-2-R also abrogated this mitogenesis, whereas an anti-CD5 mAb augmented the CD2-induced proliferation.

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