T CELLS THAT HELP B CELL RESPONSES TO SOLUBLE ANTIGEN ARE DISTINGUISHABLE FROM THOSE PRODUCING INTERLEUKIN 2 ON MITOGENIC OR ALLOGENEIC STIMULATION

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After specific activation by antigen, B cells differentiate into antibody-secreting plasma cells under the influence of a variety of factors (lymphokines) secreted by inducer/helper T cells (1-3). It is not yet clear how many lymphokines are specific for B cells but one, IL-2, has been shown to bind to both activated B cells and activated T cells (4-7), suggesting that at least one lymphokine reacts with both cell types. The role of IL-2 in B cell differentiation is undefined, but it is well established that it plays an essential role in the clonal expansion of T cells (6).

The multiplicity of lymphokines raises the question: is there functional specialization among inducer/helper T cells such that some provide differentiation factors reactive solely with B cells while others produce IL-2? Such heterogeneity would, in principle, provide a means of independently regulating the humoral and cellular components of the immune response, and would therefore have important theoretical, and possibly practical, implications.

In an earlier publication (8), Woolett et al. showed that an mAb, MRC OX-22, raised against PHA-induced rat T cell blasts, and reactive with the high molecular weight forms of the leukocyte-common antigen, revealed a phenotypic heterogeneity among rat CD4+ T cells, in that about two-thirds of these cells reacted with the antibody, and one-third did not. It was further shown (9) that the MRC OX-22- subset provided help for B cells in vivo, whereas the MRC OX-22+ population mediated graft-vs.-host reactivity and lethal GVHD. These experiments, although indicating that CD4+ T cells in the rat were functionally heterogeneous, did not provide data as to the ability of the two T cell subsets to secrete the lymphokine IL-2.

Herein, using in vitro assays for alloreactivity, IL-2 production, and B cell help, we extend the in vivo data by showing that the MRC OX-22- population, while synthesizing little or no IL-2, was very effective at providing help for in vitro antibody production. In contrast, the MRC OX-22+ subset produced as much IL-2 as unfractionated CD4+ T cells, but was relatively ineffective at inducing B cells to differentiate into antibody-secreting cells.
Materials and Methods

**Animals.** All rats were from the specific pathogen–free unit of the Medical Research Council (MRC) Cellular Immunology Unit. The strains used were PVG.RT1, PVG.RT1u (AO), and DA.RT1u, but for brevity these will be referred to as HO, HO.B2, and DA, respectively. Donors of cells used as stimulators in the mixed leukocyte cultures (MLC) were F1 hybrids of these strains. Rats were primed to DNP-bovine gamma globulin (DNP-BGG) by injecting 1 mg of alum-precipitated antigen i.p. together with 10⁹ killed Bordetella pertussis. Rats were used as thoracic duct lymphocyte (TDL) donors at least 4 wk after priming.

**Cells.** TDL were obtained by cannulation of the duct (10), and were collected at 4°C overnight into flasks containing Dulbecco’s A + B medium (DAB) and 20 U/ml heparin. Spleens were removed from rats killed by ether overdose, and the splenocytes were isolated by pressing fragments of spleen through a stainless steel mesh into ice-cold DAB containing 0.2% BSA.

**Isolation of Subsets of Lymphocytes.** Subpopulations of TDL were prepared by negative selection by the rosetting technique described by Parish and Hayward (11) and Mason (12). Briefly, TDL were incubated with mouse mAb that labelled the subpopulation(s) to be removed, then washed and mixed with SRBC coated with immunoabsorbant-purified rabbit anti–mouse Ig. The rosettes so formed were removed by differential centrifugation, and the nonrosetting cells were harvested from the supernatant. Cell fractionation was also carried out by means of a FACS II (Becton Dickinson Immunocytometry Systems, Sunnyvale, CA) using mouse mAb against rat cell membrane antigens to label the cells. Antibody-binding cells were revealed by a second incubation step with fluorescein-conjugated rabbit F(ab’)₂ anti–mouse Ig (RAM-FITC) (9). For all fractionation procedures, cell purities were assessed by FACS analysis.

**Monoclonal Antibodies.** The derivation of the mouse mAb used in this work, viz: W3/25 (anti-CD4) (13), MRC OX-8 (anti-CD8) (13), MRC OX-12 (anti-rat α chain) (14), MRC OX-6 (anti-rat I-A) (15), MRC OX-19 (13), W3/13 (pan T) (16), and MRC OX-22 (9) have all been described in the references given.

**Mixed Leukocyte Cultures.** MLC were performed as described (17) using TDL or subpopulations thereof as responders and F1 hybrid splenocytes as stimulators. The splenocytes were irradiated with 1,900 rad ¹³⁷Cs γ irradiation before culture.

**Con A Responses.** T cell subpopulations isolated from TDL were incubated, in various doses, in 96-well round-bottomed tissue culture plates with 10 µg/ml Con A, in the presence or absence of accessory cells. After 48 h in culture, [³H]thymidine was added to the wells, and incorporation of the label was determined after a further 18 h of culture. Cell doses and purities are given in Fig. 4.

**Assays for IL-2.** The IL-2 content of tissue culture supernatants was determined according to Cantrell et al. (18) by measuring the ability of these supernatants to induce [³H]thymidine incorporation in Con A–activated blasts.

**In Vitro Antibody Synthesis.** Fixed numbers of DNP-primed B cells and non-B, non-T accessory cells were plated out in 96-well round-bottomed tissue culture plates, and graded doses of DNP-BGG-primed T cells, or subpopulations of these, were added to the wells together with 10 ng/ml DNP-BGG. The accessory cells were prepared by incubating TDL with a mixture of MRC OX-8, MRC OX-12, and W3/13 mAb, and removing the labelled cells by rosetting with rabbit anti–mouse Ig–coated SRBC. After 6 d in culture, supernatants were assayed for anti-DNP antibody using a solid-phase RIA (9). The number of B cells, accessory cells, and T cells used are given in Figs. 6 and 7.

Results

**MRC OX-22 mAb Divides Rat CD4⁺ T Cells Into Negative and Weakly Positive Subsets.** Fig. 1a shows that labelling of rat TDL with MRC OX-22 antibody,
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Figure 1. MRC OX-22 mAb subdivides the CD4⁺ T cell subset. TDL, collected overnight at 4°C, were divided into two fractions. One fraction was incubated with MRC OX-22 mAb washed twice, and incubated with RAM-FITC. The other fraction was incubated with a mixture of MRC OX-12 (anti-rat κ chain) (14), and MRC OX-8 (anti-rat CD8) (13) mAb. After washing, these cells were mixed with RAM-coated SRBC, and the rosette-forming cells were (11) removed by differential centrifugation (12). The recovered non-rosette-forming cells, which were >96% W3/25⁺ (CD4⁺), were then labelled with MRC OX-22 mAb followed by RAM-FITC. All incubations were for 1 h at 4°C. After labelling, cells were examined on the FACS. Both scales are linear.

followed, after washing, by RAM-FITC, produced a trimodal fluorescence profile on the FACS. After removal of B cells and MRC OX-8⁺ (CD8⁺) cells, a bimodal profile was obtained (Fig. 1b), revealing that the W3/25⁺ (CD4⁺) cells were present in the negative and weakly positive components of the Fig. 1a profile.

Proliferation and IL-2 Production in MLC. The data in Fig. 2 show that the phenotype of the principal responding cell in the semiallogeneic MLC is OX-22⁺. The level of proliferation found in the cultures containing the MRC OX-22⁺ subset was equal to that of unfractionated TDL, an equivalent response from the MRC OX-22⁻ population requiring a fourfold greater number of cells. The use of semiallogeneic stimulators meant that effects explainable by back stimulation (17) were avoided. Since there is a requirement for CD4⁺ cells in this assay (17), it is clear that the primarily alloreactive cell in the rat MLC is CD4⁺ MRC OX-22⁺. A similar result was obtained after removing the B cells and CD8⁺ cells by rosetting before sorting on the FACS, and using the recovered MRC OX-22⁺ and MRC OX-22⁻ cells as responders in equal cell numbers (data not shown). Fig. 3 illustrates the ability of CD4⁺ MRC OX-22⁺ cells to produce IL-2 in amounts similar to those synthesized by unfractionated CD4⁺ cells. The MRC OX-22⁻ fraction, by comparison, generated far lower levels of the growth factor.

Proliferation and IL-2 Production after Lectin Stimulation. Fig. 4 shows that the majority of the proliferation observed in response to Con A by CD4⁺ cells resides in the MRC OX-22⁺ subpopulation and is accessory cell dependent. The data in
MRC OX-22 + and MRC OX-22− subpopulations of HO TDL, isolated directly by sorting on the FACS, and unfractionated TDL were cultured at varying concentrations with $5 \times 10^6$ irradiated (HO × HO.B2)F1 splenocytes as stimulators. The numbers of responders used were adjusted such that each population tested contained approximately the same number of CD4+ T cells. For example, at the highest concentration tested ($1.6 \times 10^5$ responder T cells/well), $3.72 \times 10^5$ MRC OX-22+ cells, $2.08 \times 10^5$ MRC OX-22− cells, and $3.2 \times 10^5$ unfractionated TDL were cultured. Control cultures of responders or stimulators alone incorporated no more than 1,300 cpm. Data are means and ranges of triplicate determinations. Purities were >99% for both. Cultures were carried out in 0.2-ml volumes of RPMI 1640 containing 5% DA rat serum in 96-well round-bottomed tissue culture plates with $5 \times 10^5$ irradiated (1,900 rads 137Cs γ irradiation) stimulators. After 72 h incubation at 37°C, 5% CO2, 0.5 μCi of [3H]-thymidine were added, and 18 h later, the wells were assayed for incorporation of label.

Fig. 5 show that most of the IL-2 produced by such polyclonal activation of the CD4+ subset is from the MRC OX-22+ cells, with an equal number of MRC OX-22− cells at the highest responder dose measured producing approximately fourfold less growth factor, as gauged by blast cell proliferation.

T Help for Antihapten Antibody Responses. It has been shown that CD4+ T cells in the rat provide helper activity for B cells in a primary in vitro system generating antibody against SRBC (19), whereas CD8+ cells act instead to suppress such a response. Similar results have recently been obtained using an assay system designed to measure a secondary antihapten response to DNP by DNP-BGG-primed B cells (data not shown). The CD4+ population, from DNP-BGG-primed donors, was then further fractionated on the FACS into its constituent MRC OX-22+ and MRC OX-22− subsets to determine whether a difference could be discerned in their ability to help a secondary antibody response in vitro. The data in Fig. 6 show that a marked heterogeneity in helper capacity was indeed found, with MRC OX-22− cells ~16 times as potent on a cell for cell basis.
compared with MRC OX-22+ cells in providing help for B cells to produce anti-DNP antibody. Similarly, when B cells and accessory cells plus DNP-BGG were supplemented with fixed numbers of MRC OX-22+ and MRC OX-22− cells, supernatants from the cultures containing the MRC OX-22− subsets produced >10 times as much anti-DNP antibody as those with MRC OX-22+ cells (data not shown).

**IL-2 Synthesis in Antibody-producing Cultures.** To study the association between IL-2 synthesis and the generation of B cell help, a series of cultures were set up with DNP-BGG-primed B cells, accessory cells, and MRC OX-22+ or MRC OX-22− DNP-BGG-primed CD4+ T cells. To these cultures were added either specific antigen, Con A, or medium alone. Cultures were then assayed for anti-DNP antibody on day 6 of culture, or for IL-2 on day 2 of culture with Con A stimulation, and on day 3 for the specific antigen or no antigen wells. These intervals were chosen on the basis of initial experiments that showed that IL-2 production and antibody production peaked on the days indicated.

The results in Fig. 7 confirm those illustrated in Fig. 6, in that the MRC OX-22− subset of CD4+ T cells was much more effective than the MRC OX-22+ one at providing B cell help for anti-DNP antibody responses when specific antigen was used. The figure also shows that some antibody was produced when Con A,
CD4+ T cells were isolated from HO TDL and separated into MRC OX-22+ and MRC OX-22- fractions, as described in Fig. 3, except that in the preparation of the CD4+ cells by rosette-depletion, MRC OX-6 (anti-Ia) (15) antibody was added to the mixture of MRC OX-8 and MRC OX-12 antibodies to ensure that any Ia+ cells present were also removed in the rosetting process. The MRC OX-22+ and MRC OX-22- cells were then aliquoted in the presence of Con A at 10 μg/ml in DMEM with 5% rat serum, with or without addition of 5.7 × 10^5 irradiated non-B, non-T accessory cells per well. Proliferation was assayed with an 18-h [3H]thymidine pulse after 48 h incubation. Data are means and ranges of triplicate determinations, and three separate repetitions of this experiment produced similar results. All plots have background counts with medium alone subtracted. Rosette depletion purities were 97.2% and 93.6% for CD4+ (strictly MRC OX-6-, MRC OX-8-, MRC OX-12-) cells and accessory cells (MRC OX-8-, MRC OX-12-, W3/13-), respectively. Purities for the sorted populations were: MRC OX-22+, 97.4%; MRC OX-22-, 99.7%. Closed symbols, with accessory cells added; open symbols, without accessory cells added.

rather than specific antigen, was added to the cell mixtures in culture. However, as with the wells containing specific antigen, most antibody was found in the wells containing MRC OX-22+ cells. No antibody was produced in wells containing B cells, accessory cells, and T cells but no antigen or Con A. The IL-2 assay data are shown in Fig. 8, and illustrate two points. When specific antigen was added to the wells, little IL-2 was measured even in cultures that produced the most antibody; indeed, IL-2 levels were little higher than those detected when no antigen was added. In contrast, in cultures with Con A, IL-2 production was high if the wells contained unfractionated CD4+ T cells or the MRC OX-22+ subset of these cells, but as expected, the MRC OX-22- cells produced little lymphokine.

Taking the data in Figs. 7 and 8 together, it is clear that there is a marked dissociation between IL-2 synthesis and antibody production, even when the two are assayed from the same well. The results are, however, entirely consistent with those presented in Figs. 5 and 6, which also showed that, in independent experiments, IL-2 secretion and B cell help were largely mediated by MRC OX-22+ and MRC OX-22- T cells, respectively.
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Figure 5. IL-2 production by CD4+ MRC OX-22+ and CD4+ MRC OX-22- cells after Con A stimulation. After removal of B cells, CD8+ T cells, and la+ cells by rosette depletion, the residual CD4+ cells were sorted on the FACS into MRC OX-22+ and MRC OX-22- subsets. Sorted populations were stimulated with 10 μg/ml Con A with or without 4.3 × 10⁴ irradiated non-B, non-T accessory cells, isolated from TDL, for 24 h before supernatants were harvested and tested for growth factor activity. All data are means and ranges of triplicate determinations, and have background counts with medium alone subtracted. Two further assays produced similar results. CD4+ and accessory cells were 99.3% and 90.8% pure respectively, and postsort purities for MRC OX-22+ and MRC OX-22- cells were 98% and 99%, respectively. Closed symbols, with accessory cells added; open symbols, without accessory cells added.

Discussion

Fractionation of rat CD4+ T cells using MRC OX-22 mAb, which is known to recognize the high molecular weight form of the leukocyte-common antigen, reveals striking differences in the potentials of MRC OX-22+ and MRC OX-22- subpopulations to proliferate and to secrete IL-2 upon allogeneic or nonspecific stimulation, and in their ability to facilitate antibody secretion from primed B cells during in vitro culture.

The results show that, within the CD4+ subset, it is predominantly the MRC OX-22+ cells that proliferate in response to alloantigens or T cell mitogen, and that synthesize readily detectable levels of IL-2 in either of these cases. In contrast, the MRC OX-22- cells provide optimum help for secondary antihapten antibody responses.

These results are in agreement with previous (9) in vivo observations that MRC OX-22+ cells are responsible for alloreactivity in the popliteal lymph node assay, and for causation of GVHD in irradiated F₁ hybrid rats. The finding that the helper activity for B cells resides within the CD4+ MRC OX-22+ population also agrees with the published results (9) for in vivo anti-DNP antibody production. Our data suggesting that IL-2 synthesis and B cell help are mediated by different T cell subsets are consistent with the observation (3) that some human T cell hybridomas are able to secrete B cell differentiation factor but not IL-2, and with the discovery of an immunodeficient individual whose T cells secrete...
FIGURE 6. The T helper cell for a secondary in vitro antihapten response is CD4+, MRC OX-22−. TDL from rats primed with 0.5 mg DNP-BGG ~4 wk earlier were prepared by rosette depletion into CD4+ cells (MRC OX-6−, MRC OX-8−, MRC OX-12−), B cells (W3/25−, MRC OX-8−) and accessory cells (MRC OX-8−, MRC OX-12−, W3/13−) (purities 98.1%, 98.5%, and 86.6% respectively); CD4+ cells were then sorted into MRC OX-22+ (purity, 98%) and MRC OX-22− (purity, 99.8%) fractions. Triplicate cultures containing 10 ng/ml DNP-BGG, 3.9 × 10^5 B cells, and 1.6 × 10^4 accessory cells/well, in a total volume of 300 µl DMEM plus 10% FCS were then incubated with putative helper populations, including unprimed TDL, at T cell doses ranging from 2.5 × 10^4 cells/well to 8 × 10^5 cells/well for 6 d, when 200 µl supernatant was harvested from each well. This supernatant was then tested for the presence of anti-DNP antibody in a solid-phase RIA (9). All data are the means of triplicate determinations. Control supernatant from cultures containing no T cells gave 849 cpm bound. A further experiment gave the same result. Closed symbols, cells obtained from DNP-BGG-primed rats; open symbols, cells obtained from unprimed rats.

B cell differentiation factor and B cell growth factor but not IL-2 (20). Also, Lamb and coworkers (21) were able to dissociate IL-2 synthesis from helper activity upon analysis of a panel of influenza virus–immune human T cell clones; this was found to correlate with phenotypic heterogeneity, Leu-8 (22) and 9.3 (23) mAb staining being higher in the IL-2-producing clone.

In the assays of T cell function used to examine the roles of the MRC OX-22+ and MRC OX-22− subsets of CD4+ T cells, it may be noted that the MRC OX-22− cells were active only in the assay (B cell help) that used antigen-primed cells, whereas the MRC OX-22+ cells were active in assays in which unprimed T cells were employed (MLC and mitogen responses). This use of antigen-primed T cells in some assays but not others raised the possibility that the expression or nonexpression of the MRC OX-22 antigen reflected a change in phenotype after priming rather than the existence of two independent subsets of CD4+ T cells. This suggestion is supported by the finding that T cell blasts, recovered from the rat MLC, are MRC OX-22− (J. R. Green, personal communication), although, as our data show, the T cells that respond most vigorously in this assay are initially MRC OX-22+ (whether the MRC OX-22 antigen is reexpressed when the T blasts revert to their resting state is not yet known). However, arguing against the hypothesis that the heterogeneity of MRC OX-22 antigen expression.
amongst CD4+ T cells reflects their immune status rather than their function is the observation that the MRC OX-22− subset, in contrast to the MRC OX-22+ one, responds poorly to the T cell mitogen Con A. Although the mode of action of this nonspecific mitogen is not understood, there is no evidence that it stimulates only unprimed cells. Furthermore, when the effect of adult thymectomy on the ratio of CD4+, OX-22−/CD4+, OX-22+ cells was determined, it was found to decrease (Table I). This result is opposite to that predicted from the assumption that the MRC OX-22+ subset of CD4+ T cells gives rise to long-lived memory cells that are MRC OX-22−.

A number of mAb have been described (22–26) that identify subsets of human CD4+ T cells. Although functional studies using these mAb have not been as comprehensive as those described for MRC OX-22, there are similarities between the data obtained in the two species. In particular, Morimoto and his coworkers (25, 26) have described two antibodies, 4B4 and 2H4, that label two independent subsets of CD4+ T cells. The 4B4+, 2H4− subset resembles the MRC OX-22− subset of rat CD4+ T cells in proliferating poorly to Con A but providing effective B cell help in PWM-induced Ig synthesis, whereas the 4B4−, 2H4+ subset behaves in a reciprocal fashion in these two assays, and thus functions like MRC OX-22+, CD4+ cells. The fact that the human data obtained with PWM, a
nonspecific inducer of Ig synthesis, are in accordance with the results using specific antigen in the rat supports the conclusion that MRC OX-22\textsuperscript{−}, CD4\textsuperscript{+} cells in the rat are specialized for B cell help rather than merely being memory cells.

There is presumably some functional significance in the heterogeneity of CD4\textsuperscript{+} T cells revealed herein. The fact that high-level IL-2 production can be achieved in the absence of significant levels of B cell helper activity suggests that cell-mediated immune responses may be regulated independently of antibody-mediated ones. Similarly, as potent B cell help can develop with little concomitant IL-2 production, antibody synthesis may proceed without the development of cell-mediated immunity. It is well established (27) that cytotoxic T cells (the development of which is almost certainly IL-2 dependent) can be generated against

![Graph](image-url)
minor transplantation antigens in the complete absence of a humoral response, and it is evident that many antibody responses are evoked, those against soluble antigens, for example, for which a cell-mediated response does not occur. The existence of different T cell subsets providing help for humoral and cell-mediated responses provides the possibility of regulating these two arms of the immune response independently.

Summary
An mAb MRC OX-22, reactive with the high molecular weight forms of the rat leukocyte-common antigen, has revealed a heterogeneity among CD4+ T cells in this species. Approximately two-thirds are CD4+, OX-22+, and one-third are CD4+, OX-22-. This phenotypic heterogeneity was found to be associated with a functional one. CD4+, OX-22+ cells proliferated well in mixed leukocyte culture, responded to the T cell mitogen Con A, and produced IL-2 on activation. In contrast, the CD4+, OX-22- cells performed poorly in these assays, but unlike CD4+, OX-22+ cells, did provide effective help for B cells. By sampling supernatants from cultures containing primed B cells and either of the two CD4+ T cell subsets, it was shown that, when specific antigen was included in the cultures, those containing the OX-22- subset of CD4+ cells produced high levels of antibody and some IL-2, whereas those containing the OX-22+ cells produced neither. In contrast, when specific antigen was replaced by Con A, the B cell cultures supplemented with CD4+, OX-22+ cells synthesized much higher levels of IL-2 than those containing CD4+, OX-22- cells, but only the latter cultures produced detectable levels of antibody.

The data show that inducer/helper T cells comprise two functional subsets: one that, on appropriate stimulation, synthesizes high levels of IL-2, and may therefore be presumed to play an important role in cell-mediated immunity, and another that plays an essential role in humoral responses to soluble antigens. The significance of this functional heterogeneity, with regard to the possible independent regulation of cellular and humoral responses, is briefly considered.

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