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The autonomy of CD8$^+$ T cells *in vitro* and *in vivo*

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SUMMARY

Experiments have been carried out *in vitro* and *in vivo* to determine to what extent CD8$^+$ T cells in the rat can function independently of any helper activity from CD4$^+$ cells. We have indentified the culture conditions required for the autonomous proliferation of CD8$^+$ T cells in the rat mixed leucocyte culture (MLC) and in particular have studied both the kinetics of the response and the effect of replacing the homologous serum, used in our previous MLC experiments, with fetal calf serum (FCS). The results obtained using FCS show that, early in the MLC, CD8$^+$ T-cells proliferate at a comparable rate to the CD4$^+$ subset but that, within 48–72 hr, the proliferation rate of the CD8$^+$ cells ceases to increase with time. In contrast, the proliferation of the CD4$^+$ T cells appears to be limited only by the exhaustion of the culture medium. The results also show that the proliferative responses of both CD4$^+$ and CD8$^+$ T cells are inhibited in homologous serum but that it is the CD8$^+$ subset that is more affected. When CD8$^+$ T cells, in homologous serum, are co-cultured with irradiated CD4$^+$ T cells the proliferative activity is increased, indicating that the helper activity of the CD4$^+$ T cells can over-ride the inhibitory effect of the serum. *In vivo* we have compared the abilities of injected CD4$^+$ and CD8$^+$ T cells to mediate rejection of skin allografts on nude rats. Grafts were rejected more rapidly on recipients of low doses of CD4$^+$ T cells than on rats given 200 times as many CD8$^+$ T cells. Thoracic duct lymphocytes (TDL) obtained 5 weeks after CD8$^+$ T-cell injection always contained a population of CD4$^+$ T cells, even when the injected CD8$^+$ T-cell inoculum contained less than 0·1% CD4$^+$ cells as contaminants. Evidence was obtained that these CD4$^+$ T cells found in TDL displayed alloreactivity in MLC. Further, the intentional injection of very low doses of CD4$^+$ cells led, after 5 weeks, to frequencies of CD4$^+$ T cells, in thoracic duct lymph, equal to that obtained by the injection of 200 times as many cells of the same phenotype. It appears that, in T-cell-deficient rats, CD4$^+$ cells can expand over 2000 times in a few weeks. Such expansion may explain the relatively slow rejection of skin allografts observed in these experiments when nude rats were injected with putatively pure populations of CD8$^+$ T cells. Taken together the data do not support the view that rat CD8$^+$ T cells display significant autonomy *in vivo* but they do show a generally low level of alloreactivity *in vitro*. In contrast, our results stress that CD4$^+$ T cells are uniquely potent in their ability to provide helper activity for CD8$^+$ T cells *in vitro* and to mediate graft rejection *in vivo*. Our data are therefore not consistent with the concept that the two subsets are functionally equivalent and differ only with respect to the class of MHC antigen to which they are responsive.

INTRODUCTION

In a number of studies evidence has been presented to show that the activation of cytotoxic T-cell precursors of the CD8$^+$ phenotype requires the collaboration of helper/inducer cells that express the CD4 antigen (Cantor & Boyse, 1975; Wagner & Rollinghoff, 1978; Bach *et al.*, 1977; Miller & Stutman, 1982; Mitchison & O'Malley, 1987). However, more recent experiments in both the mouse and the rat have demonstrated that CD8$^+$ T cells can be activated to alloantigens *in vitro* (Sprent & Schaefer, 1986; Rosenbert, Mizuochi & Singer, 1986; Sprent & Schaefer, 1985; Salomon *et al.*, 1984; Sopori *et al.*, 1984; Loop, Bernstein & Wright, 1980) and, in the mouse at least, can mediate allograft rejection *in vivo* (Rosenberg *et al.*, 1986; Sprent *et al.*, 1986) in the apparent absence of CD4$^+$ helper/inducer cells.

An earlier report from this laboratory (Mason, Pugh & Webb, 1981) described experiments in the rat that supported the conventional view that T cell–T cell collaboration was necessary for the *in vitro* activation of CD8$^+$ cells, and studies on skin and cardiac allograft rejection in this species led to the conclusion that CD8$^+$ T cells, acting alone, could not mediate graft

Abbreviations: FCS, fetal calf serum; [^{3}H]TdR, tritiated thymidine; LNC, lymph node cells, MHC, major histocompatibility complex; MLC, mixed leucocyte culture; TDL, thoracic duct lymphocytes.

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rejection (Dallman, Mason & Webb, 1982; Hall, de Saxe & Dorsch, 1983).

In view of the new data obtained from the work in the mouse and rat, we have re-examined the question of the helper cell dependence of CD8$^+$ cells in the rat both *in vitro* and *in vivo*. The results are discussed with reference to disparate data obtained from similar studies in these species.

MATERIALS AND METHODS

Animals

All euthymic rats were from the specific pathogen-free unit of this laboratory. The following strains were used: PVG (RT1^c), PVG.RT1^u, DA (TR1^a), AO (RT1^u) and Lew (RT1^l). Congenitally athymic nude rats, PVG rnu/rnu, were from Harlan Olac Ltd, Bicester, Oxon.

Cells

Responder cells were thoracic duct lymphcytes (TDL) collected overnight at 4° following cannulation of the duct by a standard procedure (White *et al.*, 1978).

Preparation of T-cell subsets

TDL were depleted of various subpopulations by incubating the cells, at 4°, with appropriate mouse monoclonal antibodies or mixtures thereof (see below), washing the cells and mixing them with sheep erythrocytes coated with immunoabsorbent-purified rabbit anti-mouse Ig antibody. The aggregates of erythrocytes and lymphocytes labelled by the mouse monoclonal antibodies were spun out by a brief centrifugation step and the supernatant, containing the non-rosette-forming lymphocytes, was recovered. Any residual sheep erythroctyes were removed by hypotonic lysis and the purity of all cell preparations was checked by flow cytofluorography (White *et al.*, 1978). Details of the rosetting process are described more fully elsewhere (Mason, Penhale & Sedgwick, 1987).

Stimulator cells

Single cell suspensions of splenocytes or lymph node cells were prepared by gently pressing the organs through a stainles steel mesh using the plunger of a 5-ml syringe. Debris was removed by filtration through a loose plug of cotton wool and the cells were washed twice before use. They were irradiated with 2000 rads ^{137}Cs irradiation, a dose which virtually eliminated their ability to act as responders but left their stimulatory activity unimpaired.

Monoclonal antibodies

All antibodies were in the form of tissue culture supernatant and were of mouse origin. They were: MRC OX6 (anti-rat class II MHC antigen; McMaster & Williams, 1979), MRC OX12 (anti-rat kappa chain; Brideau *et al.*, 1980), W3/25 and MRC OX35 (anti-rat CD4 antigen; White *et al.*, 1978; Jeffries, Green & Williams, 1985), MRC OX8 (anti-rat CD8 antigen; Brideau *et al.*, 1980), MRC OX1 (anti-leucocyte common antigen; Woollett *et al.*, 1985), MRC OX39 (anti-rat IL-2 receptor; Paterson *et al.*, 1987) and MRC OX40 (antigen expressed only on activated CD4$^+$ T cells; Paterson *et al.*, 1987). To prepare CD4$^+$ T cells from TDL the latter were incubated with a mixture of MRC OX6 or MRC OX12 and MRC OX8 monoclonal antibodies before rosetting, while to obtain CD8$^+$ T cells a mixture of MRC OX6 or MRC OX12, W3/25 and MRC OX35 antibodies was used. The MRC OX6 antibody removed both B cells and any class II MHC antigen-positive dendritic cells.

Flow cytofluorographic analysis of TDL

TDL collected overnight at 4° were labelled with the monoclonal antibodies given in the Results and analysed for surface phenotype using a Becton-Dickinson FACS II cell sorter. The procedure is described in Mason, Penhale & Sedgwick (1987).

Media

Mixed leucocyte cultures (MLC) were set up using RPMI-1640 supplemented with 2 mM glutamine, $2{\cdot}5 \times 10^{-5}$M 2-mercaptoethanol and antibiotics. Serum, heat-inactivated at 56° for 30 min, was either fetal calf serum (FCS) or DA rat serum. The homologous serum was added to the RPMI in various concentrations and, in some experiments, mixtures of DA serum and FCS were used. Details are given in the Results. Prior to setting up the MLC, cells were handled in phosphate-buffered saline containing 0·2% bovine serum albumin and were kept at 4°.

Mixed leucocyte cultures

Responder cells and irradiated stimulators were cultured together in 96-well round-bottomed plates for 24, 48 or 72 hr at 37° and then pulsed, for 6 hr or 18 hr, with 0·5 μCi tritiated thymidine. Cells were then harvested and assayed for incorporation of the radiolabel. Stimulator cell doses, except where noted, were held constant at 5×10^5/well; responder cell doses are given in the Results. In some experiments, anti-CD4 monoclonal antibody (W3/25), in the form of purified IgG, was added to the MLC at a final concentration of 1–10 μg/ml. All cultures were carried out in triplicate.

Skin grafts

Athymic nude rats of PVG strain were grafted on the left and right flanks with full thickness skin grafts from DA, AO or Lew donors. Bandages were removed on Day 7 and by Day 13 all grafts had healed in and were cosmetically perfect. At this time the rats were injected i.v. with CD4$^+$ or CD8$^+$ cells. Cell doses are given in the Results.

RESULTS

CD8$^+$ cells respond in the MLC in homologous serum when co-cultured with CD4$^+$ cells

In order to confirm our previous findings (Mason *et al.*, 1981), that CD8$^+$ T cells can be induced to proliferate in 5% homologous serum if CD4$^+$ helper/inducer cells are also present, cultures were set up with PVG strain CD8$^+$ cells as responders and irradiated DA splenocytes as stimulators. Irradiated PVG strain CD4$^+$ T cells were added to some wells as a source of T-cell help. Table 1 shows that the weak autonomous proliferative response of the CD8$^+$ cells was markedly augmented by the addition of the irradiated CD4$^+$ T cells, although the latter were not themselves proliferating. These results indicated that homologous serum would support the proliferation of CD8$^+$ T cells but that these cells did not undergo vigorous autonomous proliferation in this medium, at least at the responder cell dose used.

Table 1. CD8+ T cells proliferate in the MLC in homologous serum if provided with help from CD4+ T cells

Responder cells present in culture	Stimulator spleen cells	C.p.m.*
$1{\cdot}25\times10^5$ CD8+ T cells†	DA‡	18,500
$1{\cdot}25\times10^5$ CD8+ T cells + 5×10^5 irradiated CD4+ T cells	DA	64,000
5×10^5 irradiated CD4+ T cells alone§	DA	1000
$1{\cdot}25\times10^5$ irradiated CD8+ T cells + 5×10^5 non-irradiated CD4+ T cells	DA	145,000
5×10^5 non-irradiated CD4+ cells alone	DA	176,000

* 72-hr culture plus 18-hr pulse of tritiated thymidine. In this experiment and all others all c.p.m. are means of triplicate determinations.

† PVG strain TDL were depleted of CD4+ T cells and B cells by rosetting using W3/25, MRC OX35 and MRC OX6 monoclonal antibodies. The depletion reduced the percentage of cells reactive with these antibodies from 89·5 to 1·3%. Of the recovered, depleted cells, 73% were CD8+ and the remaining 26% were null cells.

‡ Irradiated with 2000 rad ^{137}Cs gamma irradiation.

§ PVG TDL were depleted of CD8+ T cells and B cells using a mixture of MRC OX8 and MRC OX6 antibodies to label the TDL before rosetting. Predepletion 61·8% cells labelled, postdepletion 0·6%. The recovered cells were 93% CD4+ T cells. The cells were irradiated with 950 rad ^{137}Cs gamma irradiation.

Table 2. Failure to induce strong autonomous proliferation of CD8+ T cells at high cell dose and evidence for their partial inhibition of CD4+ T-cell proliferation

PVG strain T cells present in culture*	Anti-CD4 antibody†	C.p.m.‡
$1{\cdot}25\times10^5$ CD8+	—	18,500§
5×10^5 CD8+	—	24,500
5×10^5 CD8+ + 5×10^5 CD4+	—	94,000
5×10^5 CD8+ + 5×10^5 CD4+	+	44,000
5×10^5 CD4+	—	182,000
5×10^5 CD4+	+	8300

* Cells were from the same preparation as that in Table 1. Stimulator population was 5×10^5 irradiated DA strain splenocytes.

† Final concentration 2·5 µg/ml of W3/25 monoclonal antibody.

‡ 72-hr culture + 18-hr pulse of tritiated thymidine.

§ Data from Table 1.

To see whether an increase in the dose of CD8+ T cells would show better autonomous proliferation (a result that might be expected in view of the fact that activated T cells produce their own growth factors) an MLC was set up with 5×10^5 CD8+ T cells/well. As Table 2 indicates, this number of CD8+ T cells responded hardly more vigorously than did the smaller dose in Table 1 and an equal number of CD4+ T cells incorporated over seven times as many counts/min. Mixing the CD4+ and CD8+ T cells gave an intermediate level of proliferation—possibly because the CD8+ cells were having an inhibitory effect on the CD4+ cells or possibly because they were simply consuming the growth factors produced by them. Additional data on this problem are presented later in the paper.

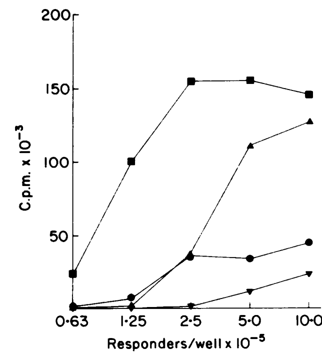

Figure 1. The inhibitory effect of homologous serum on the proliferative responses of CD4+ and CD8+ T cells in the rat MLC. TDL of AO strain were fractionated into CD4+ and CD8+ subsets by rosette depletion using appropriate mixtures of MRC OX8 and MRC OX12, or W3/25, MRC OX35 and MRC OX12 monoclonal antibodies to label the cells. Stimulators were DA strain splenocytes, irradiated with 2000 rads ^{137}Cs gamma irradiation, and used at 10^6 cells/well. Tissue culture medium was RPMI-1640 containing 5×10^{-5}M 2-ME and antibiotics together with 5% FCS or 5% DA serum. A second experiment, run in parallel employed a different batch of DA serum. Culture was for 72 hr with an 18-hr pulse of [^{3}H]TdR. (■——■), CD4+ cells in FCS; (▲——▲), CD4+ cells in serum; (●——●), CD8+ cells in FCS; (▼——▼), CD8+ cells in DA serum.

Effect of replacing homologous serum with FCS

There are a number of reports that rodent sera inhibit T-cell activation *in vitro* (Lelchuk & Playfair, 1985; Hardt *et al.*, 1981; Nelson & Schneider, 1974), and in some instances this inhibition has been attributed to the presence of an IL-2 antagonist (Lelchuk & Playfair, 1985). As Table 1 and the data in Mason *et al.* (1981) show, this inhibitory activity of homologous serum, if present, can be overcome in MLCs containing CD8+ responder cells, by the addition of irradiated CD4+ T cells. It is probable that the CD4+ cells augment any IL-2 produced by the CD8+ cells themselves and thus induce the latter cells to proliferate. MLC were set up, therefore, using FCS in place of homologous serum to see whether, in the absence of any putative anti-IL-2 activity, the CD8+ cells would undergo vigorous autonomous proliferation.

The results, illustrated in Fig. 1, show two characteristics: first FCS increased the proliferative capacity of both CD4+ and CD8+ T-cell subsets when it was used in the place of rat serum. [A further experiment, using a different batch of homologous serum gave a virtually identical result (data not shown).]

Second, whereas the dose–response curve, in FCS, for both the CD4+ and CD8+ subsets showed a plateau in the response for cell doses in excess of $2{\cdot}5\times10^5$ per well, the level of the plateau was much lower for the CD8+ cells (35,000 c.p.m.). The incorporation of radiolabel by the CD4+ cells is close to the maximum achievable in our culture system before pH changes in the media have adverse effects on cell activity. However, no such culture effect could explain the low plateau obtained with the CD8+ subset. Table 3 shows that the levelling off of the response of CD8+ cells with increasing dose was not confined to a single strain combination and, with the strains used in Table 3, the plateau developed at a responder dose of $1{\cdot}25\times10^5$ cells/well. In this experiment anti-CD4 monoclonal antibody was added to some of the cultures to confirm that the CD4+ T-cell cultures, but not those containing the CD8+ subsets, could be inhibited

Table 3. Biphasic dose–response curve for $CD8^+$ T cells in the PVG-responder/DA-stimulator strain combination

Responder cell phenotype	Responder cells/well $\times 10^{-5}$	C.p.m. Without anti-CD4 antibody	C.p.m. With anti-CD4 antibody
$CD8^+$	0·31	1800	3800
	0·62	15,000	14,000
	1·25	78,600	69,000
	2·50	60,000	71,000
	5·00	60,600	53,000
$CD4^+$	0·31	5500	700
	0·62	30,000	2000
	1·25	135,000	5000
	2·50	192,000	14,000
	5·00	196,000	44,000

Irradiated stimulator splenocytes were at 5×10^5/well in all cases. Anti-CD4 antibody was added to some wells at a final concentration of 10μg/ml. Culture was for 72 hr with an additional 18 hr of culture with tritiated thymidine. The culture medium contained FCS.

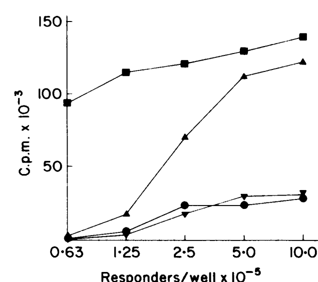

Figure 2. Failure of rat $CD8^+$ T cells to respond vigorously in the MLC using allogeneic LNC as stimulators. AO strain TDL were separated, by negative selection, into $CD4^+$ and $CD8^+$ subsets as described in Fig. 1. Removal of the appropriate subset was more than 99·5% complete in both cases. Stimulators were cervical lymph node cells from DA rats and were used at 5×10^5 cells/well after irradiation with 2000 rads. The culture medium was also as in Fig. 1 and contained 5% FCS. Some wells contained anti-CD4 antibody (W3/25) at a final concentration of 2 μg/ml. The culture period was for 72 hr followed by an 18-hr pulse of [^{3}H]TdR. (■——■), $CD4^+$ cells in medium; (▲——▲), $CD4^+$ cell in anti-CD4 antibody; (●——●), $CD8^+$ cells in medium; (▼——▼), $CD8^+$ cells in anti-CD4 antibody.

by this antibody. Failure to inhibit the cytotoxic/suppressor T-cell cultures confirmed that the observed proliferation did not depend on helper activity provided by the few contaminating helper/inducer cells.

It has been reported that rat splenocytes, but not rat lymph node cells, contain a population of macrophages that inhibits the MLC (Oehler *et al.*, 1977; Weiss & Fitch, 1977). In these experiments it appears that only macrophages in the responder population were able to mediate the suppressive effect since the removal of adherent cells from the responders abrogated the suppression, even when unfractionated allogeneic splenocytes were used as stimulators. However, to confirm that the poor proliferative responses of $CD8^+$ T cells in the rat MLC were not due to the inhibitory effect of any splenocytes in the cultures, MLCs were set up using allogeneic LNC as stimulators and $CD8^+$ T cells, isolated from TDL, as responders. As Fig. 2 shows, the $CD8^+$ T cells responded no better in this system than they did when splenocytes were used as stimulators.

Time–course of proliferation for the $CD4^+$ and $CD8^+$ T-cell subsets in the MLC

The anomalous dose–response characteristics of proliferation of $CD8^+$ T cells, described in the preceding section, suggested that these cells were limiting their own activation at high cell numbers. The ability of rat $CD8^+$ T cells to inhibit the MLC has been well documented (Salomon *et al.*, 1984) and a similar action of mouse $CD8^+$ cells has been described (Cantor, Shen & Boyse, 1976). In both series of experiments inhibitory activity was assayed in cultures of longer than 72 hr duration, i.e. late in the culture period compared with the experiments described here. However, to study the possibility of autoregulation of proliferation, by the $CD8^+$ T cells, MLCs were set up for different culture periods. Figure 3 shows the result of such an experiment using FCS in the culture medium. At 24 hr the $CD4^+$ and $CD8^+$ T-cell subsets incorporated low, but equal, levels of [^{3}H]TdR. By 48 hr there was a clear separation with a shallower

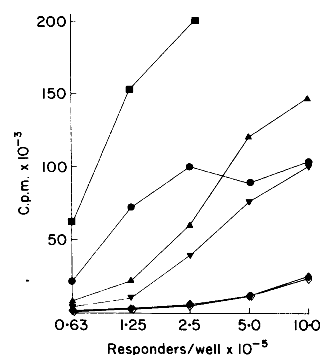

Figure 3. Proliferation of $CD4^+$ and $CD8^+$ T cells measured at different times after initiation of the MLC. Responders and stimulators were prepared as for Fig. 1 except that the latter were used at 5×10^5 cells/well. After depletion the $CD8^+$ T cells contained less than 0·9% $CD4^+$ cells and the $CD4^+$ T cells were contaminated with less than 0·3% $CD8^+$ cells. Cultures were for 24, 48 or 72 hr followed by an 18-hr pulse of [^{3}H]TdR. (♦——♦), $CD4^+$ T cells 24 hr; (◊——◊), $CD8^+$ T cells 24 hr; (▲——▲), $CD4^+$ T cells 48 hr; (▼——▼), $CD8^+$ T cells 48 hr; (■——■), $CD4^+$ T cells 72 hr; (●——●), $CD8^+$ T cells 72 hr.

dose–response curve for the $CD8^+$ population compared to the $CD4^+$ one. At 72 hr the dose–response curve of the $CD8^+$ subset was markedly non-linear with a plateau starting at a cell dose of $2·5 \times 10^5$ cells/well. The results suggest that autoregulation does occur and can be detected as early as 48 hr after culture is started.

The failure to find evidence for autonomy of $CD8^+$ T cells *in vivo*

As the results in Fig. 1 show, homologous serum had a strong inhibitory effect on the ability of rat $CD8^+$ T cells to respond autonomously in the MLC. Bearing in mind that this inhibition was demonstrable with rat serum at 5% of its *in vivo* concentra-

Table 4. Comparison of the capacity of $CD4^+$ and $CD8^+$ T cells to mediate skin allograft rejection*

	Rat no.	T cells injected	Strain of skin graft donor	Day of complete graft rejection
Exp. 1	1	1×10^7	DA	42
		$CD8^+$	AO	25
	2	1×10^7	DA	32
		$CD8^+$	Lew	25
	3	1×10^7	AO	44
		$CD8^+$	Lew	43
	4	1×10^7	DA	14
		$CD4^+$	AO	14
	5	1×10^7	DA	13
		$CD4^+$	Lew	10
	6	1×10^7	AO	14
		$CD4^+$	Lew	10
Exp. 2	1	1×10^7	DA	26
		$CD8^+$	AO	27
	2	1×10^7	AO	39
		$CD8^+$	Lew	32
	3	1×10^7	DA	12
		$CD4^+$	AO	13
	4	1×10^7	AO	13
		$CD4^+$	Lew	11
	5	5×10^4	DA	23
		$CD4^+$	Lew	18
	6	5×10^4	DA	23
		$CD4^+$	Lew	18

* Data from two independent experiments are presented. For the first experiment (first set of six animals) athymic PVG rats grafted on the right and left flanks 13 days earlier with skin grafts from the donor strains indicated, were injected, i.v., with 1×10^7 $CD4^+$ or $CD8^+$ T cells isolated from PVG strain TDL by rosette depletion. The second experiment (second set of six rats) was identical to the first except for the reduced dose of $CD4^+$ T cells for Rats 5 and 6.

tion it might be anticipated that $CD8^+$ T cells would not readily respond to alloantigens *in vivo*. To study this point PVG strain athymic rats were given full thickness skin grafts from MHC incompatible donors and 13 days later, when the grafts were well established, the recipients were injected i.v. with 1×10^7 $CD4^+$ or $CD8^+$ PVG strain T cells.

The results, in Table 4, Exp. 1, demonstrate that grafts on rats given $CD4^+$ T cells were rejected in a prompt and clear-cut manner. In contrast, grafts on recipients of $CD8^+$ T cells were rejected much later. In earlier experiments, T-cell deficient rats, injected with highly purified inocula of $CD8^+$ T cells, usually failed to reject their skin allografts (Dallman *et al.*, 1982). However, the $CD8^+$ T-cell inocula used in the present experiments contained approximately 0·3% $CD4^+$ T cells as contaminant and, in consequence, each nude rat given 1×10^7 $CD8^+$ T cells also received 3×10^4 cells of $CD4^+$ pheontype. Note that these figures represent the maximum levels of contamination, since some of the residual cells that escaped the depletion procedure may have been B cells and these would be detected in the post-depletion purity checks. It has been reported that $CD4^+$ T cells, injected into syngeneic nude rats, undergo many rounds of clonal expansion (Bell *et al.*, 1987) and, to determine whether such an expansion was occurring in the skin graft experiments, TDL were collected and analysed for surface phenotype using flow cytofluorography. As Table 5 (rows 1 and 2) and Fig. 4 show, within 5 weeks of injecting a nude rat with an inoculum of 1×10^7 $CD8^+$ T cells and a maximum of 3×10^4 $CD4^+$ T cells the two subsets were represented with approximately equal frequency, implying an approximately six-fold expansion for the cytotoxic/suppressor T-cell subset but at least a 2×10^3-fold expansion for the helper/inducer one. One of the rats given the $CD8^+$ T-cell inoculum had rejected one of its two allografts by the day of cannulation but the other had rejected neither (Rats 1 and 3 of Table 4, Exp. 1). In contrast the rat given $CD4^+$ cells (Rat 4) had rejected both.

To determine whether very small numbers of $CD4^+$ T cells, injected into nude rats, could mediate skin allograft rejection the skin graft experiment was repeated using an inoculum of 5×10^4 cells of this phenotype. The results of this experiment (Exp. 2, Table 4) demonstrate the potency of $CD4^+$ T cells in this assay in that recipients of these cells rejected their allografts more promptly than rats given two hundred times as many $CD8^+$ T cells.

In this experiment a slight modification of the rosette depletion procedure was adopted in the preparation of the $CD8^+$ cells. TDL were incubated with a mixture of MRC OX6 antibody (to label the B cells) and biotin-conjugated anti-CD4 antibody before mixing with the rabbit anti-mouse Ig-sensitized sheep erythrocytes. To check for completeness of depletion of the $CD4^+$ cells a sample of the non-rosette forming fraction was incubated with phycroerythrin-conjugated avidin and examined on the FACS. No contaminating cells of $CD4^+$ pheonotype were detected in this sample. Given that the threshold of detection on the FACS analysis is approximately 0·1%, this result implied that rats given 10^7 $CD8^+$ T cells received less than 10^4 $CD4^+$ T cells. However, the marked proliferative capacity of $CD4^+$ T cells *in vivo*, as indicated by the data of Table 5, and the ability of small inocula of such cells to mediate graft rejection, raised the question of whether undetectable levels of $CD4^+$ T cells, contaminating the inocula of $CD8^+$ cells, could play a role in skin graft rejection in these experiments. To approach this question, TDL, of Rats 1, 3 and 5 of Exp. 2, Table 4, were collected 5 weeks after the injection of donor cells and set up in MLCs using allogeneic splenocytes as stimulators. The spleen cell donors were chosen to be either syngeneic with the skin graft donors or to be third party. To assess whether the proliferative activity in these MLCs was dependent on $CD4^+$ T cells, anti-CD4 antibody was included in some of the culture wells. In addition some of the TDL were subject to phenotype analysis on the FACS and these data are shown in the second part of Table 5.

The results from the MLCs are illustrated in Fig. 5. It is evident that TDL from rats given either 5×10^4 or 10^7 $CD4^+$ T cells responded well in the MLC to all strains of stimulator splenocytes and that most of the proliferation observed was inhibitable by anti-CD4 antibody included in the cultures (compare Fig. 5 with Fig. 2). In contrast, the TDL from the rat given 10^7 $CD8^+$ T cells proliferated less well and the degree of inhibition with anti-CD4 antibody, while clearly significant, was generally less marked, an exception being the response to Lew splenocytes where almost complete inhibition was obtained. We have never observed partial inhibition by anti-CD4 antibody of an MLC containing purified $CD8^+$ responder T cells (e.g. see Fig. 2 and Table 3), so the partial effect seen with the TDL

Table 5. Surface phenotype of TDL recovered from nude rats given subsets of syngeneic T cells 35 days before analysis

Rat no.	T cells injected	% lymphocytes of indicated phenotype*					
		sIg^{+}†	$CD5^{+}$	$CD4^{+}$	$CD8^{+}$	$IL\text{-}2R^{+}$‡	$OX40^{+}$§
1¶	$10^{7}CD8^{+}$	64·2	22·7	11·2	12·4	6·6	7·5
3	$10^{7}CD8^{+}$	74·2	10·7	4·8	7·3	ND	4·5
4	$10^{7}CD4^{+}$	67·3	20·5	22·5	1·7	5·1	6·5
1**	$10^{7}CD8^{+}$	ND	18·7	8·8	8·7	ND	5·2
3	$10^{7}CD4^{+}$	65·6	24·0	19·6	0·5	ND	7·1
5	$5\times10^{4}CD4^{+}$	73·3	20·6	22·1	3·6	ND	10·6
Control nude	0	83·3	0·6	2·2	1·7	1·0	ND

* TDL was obtained 5 weeks after injection of the T-cell inocula.
† Surface immunoglobulin positive.
‡ Interleukin-2 receptor positive.
§ MRC OX40 antigen is expressed only on activated $CD4^{+}$ T cells (Paterson *et al.*, 1987). Note that the total of sIg^{+} cells + $CD5^{+}$ cells is less than 100% in all cases, indicating a significant population of null cells. Such a result has been reported previously (Bell *et al.*, 1987).
¶ Rats 1, 3 and 4 are those of the first skin graft experiment (Table 4, Exp. 1).
** Rats 1, 3 and 5 derive from the second skin graft experiment (Table 4).

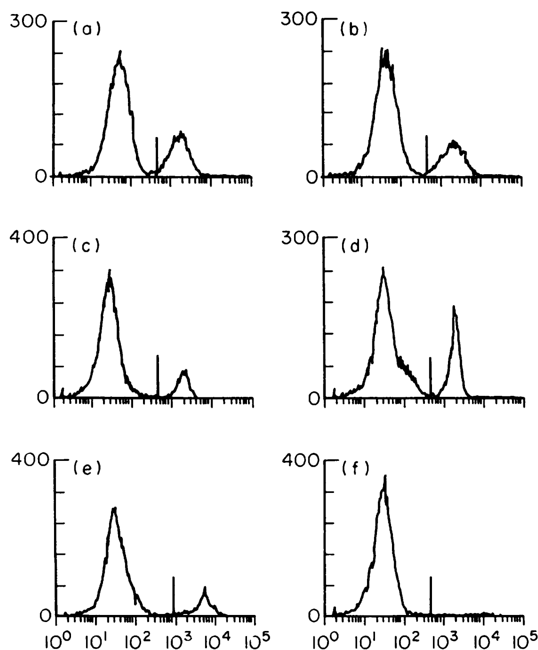

Figure 4. Flow cytofluorographic analysis of TDL from nude rats given syngeneic T cells. Athymic rats were given inocula of syngeneic T cells containing either 1×10^{7} $CD8^{+}$ cells + 3×10^{4} $CD4^{+}$ cells [traces (a), (c) and (e)], or 1×10^{7} $CD4^{+}$ cells + 3×10^{4} $CD8^{+}$ cells [traces (b), (d) and (f)]. Five weeks later the thoracic ducts of the two rats were cannulated and samples of the TDL were incubated with mouse anti-rat monoclonal antibodies followed by fluorescein-conjugated rabbit anti-mouse Ig. Traces (a) and (b), CD5 label; (c) and (d), CD4; (e) and (f), CD8. The TDL for traces (a), (c) and (e) were obtained from Rat 1 Exp. 1 of Table 4. It had rejected its AO graft but not its DA graft at this time. Rat 4 Exp. 1 of Table 4 provided the TDL for traces (b), (d) and (f). Both of its grafts had been rejected 3 weeks before cannulation of its duct.

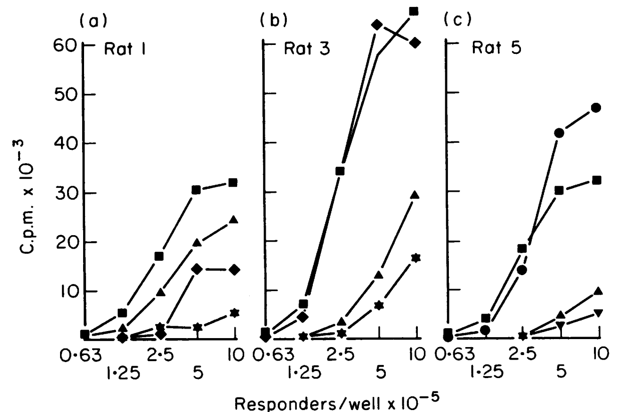

Figure 5. Proliferation, in the MLC recovered from T-cell subset-reconstituted athymic rats. Athymic rats, given allogeneic skin grafts 13 days previously were injected i.v. with 10^{7} syngeneic $CD8^{+}$ T cells (Rat 1 Exp. 2) or 10^{7} $CD4^{+}$ T cells (Rat 3 Exp. 2) or 5×10^{4} $CD4^{+}$ T cells (Rat 5) and their thoracic duct lymphocytes collected 34 days after cell injection. Mixed lymphocyte cultures, using irradiated AO, DA and Lew splenocytes as stimulators, were set up in culture medium with 5% FCS. Culture was for 72 hr with an 18-hr [^{3}H]TdR pulse. Anti-CD4 monoclonal antibody, at 2 μg/ml final concentration, was added to some wells. The skin graft survival times of the TDL donors are given in Table 4, Exp. 2 and the surface phenotype analysis in Table 5. (■——■), response to DA splenocytes, no anti-CD4 antibody in culture; (▲——▲), response to DA splenocytes, with anti-CD4 antibody in culture; (♦——♦), response to Lew splenocytes, no antibody in culture; (★——★) response to Lew splenocytes, with anti-CD4 antibody in culture; (●——●) response to AO splenocytes, no anti-CD4 antibody in culture; (▼——▼), response to AO splenocytes, with anti-CD4 antibody in culture.

responders from the nude rat given $CD8^+$ T cells suggests that some $CD4^+$ T cells were also present in the responder population. The phenotypic analysis (Table 5, row 4) of this TDL supports this conclusion in that almost 9% of the cells were $CD4^+$. Virtually the same number reacted with anti-CD8 antibody but these latter cells were not also $CD4^+$ since a mixture of anti-CD4 and anti-CD8 antibodies labelled 17·4% of TDL, a number very close to the expected value of 17·3% if the $CD4^+$ and $CD8^+$ subsets were mutually exclusive.

DISCUSSION

The present experiments extend and modify the conclusions contained in our previous publication (Mason *et al.*, 1981), but they do not lead to a radical change in our view of the interactions between $CD4^+$ and $CD8^+$ T cells in the rat. By substituting FCS for homologous serum in the MLC we can clearly show that $CD8^+$ T cells will proliferate in the absence of any helper activity from the $CD4^+$ subset. The data demonstrate that homologous serum has an inhibitory action on both the $CD8^+$ and the $CD4^+$ subsets in the MLC and it is the cytotoxic/suppressor subset that is the more affected (Fig. 1). In fact, as Table 2 shows, $CD8^+$ T cells proliferated poorly in homologous serum but could be induced to respond more vigorously when provided with helper activity from irradiated $CD4^+$ cells (Table 1). This finding is in accord with our previous data (Mason *et al.*, 1981).

The replacement of homologous serum, in the MLC cultures, with FCS not only demonstrated the autonomous proliferation of $CD8^+$ T cells but also indicated that, even under these conditions, they responded less well than the $CD4^+$ subset. Using a culture period of 72 hr with an 18-hr tritiated thymidine pulse it was found that above a critical cell dose in the range $1{\cdot}2 \times 10^5$ responders/well, the level of [^{3}H]TdR incorporation did not increase with increasing cell dose but either developed a plateau or even declined slightly (Figs 1 and 2 and Table 3). This effect, which was most apparent at 72 hr of culture was already detectable at 48 hr when the gradient of the dose–response curve for the $CD8^+$ cells was clearly shallower than for the $CD4^+$ subset (Fig. 3), whereas the gradients at 24 hr were virtually identical. Such a result might, in principle, be accounted for if the $CD8^+$ T-cell subset needed larger numbers of stimulator cells for optimum activation than did the $CD4^+$ subset. However, no such differential requirement for stimulator cells has been observed in the mouse MLC (Inaba, Young & Steinman, 1987). It was also evident that this apparent autoregulation of the $CD8^+$ T cells was not confined to this subset but also partially suppressed the proliferative response of the $CD4^+$ T cells (Table 2). As noted in the Results, these findings are consistent with other data in the rat (Salomon *et al.*, 1984) that show that $CD8^+$ cells, when activated, can inhibit MLC. Noting that the inhibitory activity of $CD8^+$ T cells becomes stronger as the duration of the MLC increases, the most reliable measure of the ability of these to respond to allogeneic stimulation is probably obtained at very early culture times. Given that at 24 hr of culture $CD8^+$ and $CD4^+$ T cells proliferated equally well (Fig. 3), we may conclude that the alloreactivity of the two subsets is very similar when assayed in this way.

It should be emphasized that this result may be valid only for cultures containing FCS rather than homologous serum and the data obtained using the heterologous source of serum are clearly of questionable relevance. Indeed, given that even 5% homologous serum had such a significant effect on the ability of T cells to proliferate in the MLC, one may question whether any of the *in vitro* data have relevance to the responses of T cells *in vivo*.

Three other groups have reported the autonomous proliferation of $CD8^+$ T cells in the rat MLC. In two of these (Salomon *et al.*, 1984; Loop *et al.*, 1980), the maximum proliferative response of the $CD8^+$ T cells was markedly less than that of the $CD4^+$ subset, in agreement with our own findings. The data from the third group (Sopori *et al.*, 1984) conflict with these results in that the T-cytotoxic/suppressor and T-helper subsets proliferated equally well. Differences in the detailed experimental procedures may account for these variations but the essential differences are not apparent.

In the present series of experiments, several assays were made of IL-2 production by $CD8^+$ T cells proliferating in mixed leucocyte cultures containing FCS. However, attempts to compare the levels detected with those produced by cultures containing $CD4^+$ T cells were frustrated by the fact that the dose–response curves (i.e. the variation in the amount of IL-2 detected with the number of responder T cells added in the MLC) were not parallel for the two T-cell subsets. The dose–response curves for the $CD8^+$ subset were always very shallow, possibly reflecting either very rapid consumption of IL-2 or the presence of an inhibitory factor that interfered with the interleukin assay (data not shown). The presence of such an inhibitor might be anticipated from the fact that the $CD8^+$ T-cell subset showed autoregulation of its proliferation in the MLC. In two published reports, in which IL-2 production from $CD4^+$ and $CD8^+$ T cells has been studied in the rat, none of the lymphokine was detected in the supernatants of the $CD8^+$ T cells (Salomon *et al.*, 1984; Cantrell, Robins & Baldwin, 1982). These failures to detect IL-2 in cultures of proliferating rat $CD8^+$ T cells contrasts with experiments with mouse cells where the lymphokine was readily detected (Inaba *et al.*, 1987).

The results of the skin allograft experiments (Table 4) demonstrate that, compared with $CD4^+$ T cells, the $CD8^+$ subset is markedly deficient in its ability to mediate graft rejection, at least in the three strain combinations studied. Specifically 5×10^4 $CD4^+$ T cells produced more rapid graft rejection then 10^7 $CD8^+$ cells. The interpretation of these skin graft experiments (with regard to the possible autonomy of the $CD8^+$ T-cell subset) is complicated by the fact that $CD4^+$ T cells, injected into a syngeneic rat, undergo vigorous proliferation (Bell *et al.*, 1987). As Table 5 shows, $CD4^+$ T cells were always detectable in the TDL of athymic rats given purified $CD8^+$ cells, even when the donor inocula contained no measurable contaminant of $CD4^+$ cells. Whether this $CD4^+$ T-cell population was sufficient to provide significant help for the $CD8^+$ T cells is not a question that can be answered by the present experiments but the MLC data (Fig. 5) definitely raise this possibility. Nor is it known whether these $CD4^+$ cells can themselves mediate the rejection of the skin allografts on nude rats given nominally pure $CD8^+$ T cells. Similar studies of the rejection of skin allografts in mice seem to have avoided this problem since in those experiments no significant expansion of $CD4^+$ T cells was observed (Rosenberg, Mizuochi & Singer, 1986). However, a series of previous experiments, where T-cell deficient rats have been injected with syngeneic $CD8^+$ T cells containing 0·27% or less $CD4^+$ cells as contaminants, only rats receiving the more highly contaminated inocula rejected their

skin allografts (Dallman *et al.*, 1982). Furthermore, as noted in the Introduction, CD4+, but not CD8+, T cells are able to mediate rat cardiac allograft rejection (Hall *et al.*, 1983). These earlier data, taken together with the current ones, lend little support to the view that rat CD8+ T cells act autonomously *in vivo* to mediate allograft rejection.

As Table 5 shows, the TDL of the nude rats injected with T cells from euthymic donors contained a high frequency (5–7%) of cells expressing Il-2 receptors. Such cells are almost undetectable in TDL from normal rats. The frequency of cells expressing the MRC OX40 antigen (a marker for activated CD4+ T cells; Paterson *et al.*, 1987) was similar to that of IL-2 receptor-positive cells, indicating that it was the CD4+ subset that was activated. In an earlier publication we reported on the ability of purified CD8+ T cells to mediate lethal graft-versus-host disease (Mason, 1981). As noted in that publication, in interpreting those experiments we could not exclude the possibility that the very small number of CD4+ T cells (less than 0·2%) that contaminated the CD8+ inocula played an essential role by providing helper activity for the latter cells, and this problem is intrinsic to all experiments of this type. However, there is good evidence that CD8+ T cells in the mouse may autonomously mediate lethal graft-versus-host disease (Sprent *et al.*, 1986). In those experiments the existence of class I MHC mutants was employed to develop graft-versus-host disease in a strain combination in which CD8+, but not CD4+, T cells were able to mediate disease. In such experiments it seems unlikely that any CD4+ T cells, of either host or donor origin, that survived the procedures to eliminate them, could have played an active role. Despite our difficulties in demonstrating allograft rejection by CD8+ T cells in the rat it may be that such cells do in fact demonstrate more autonomy in mediating graft-versus-host disease than they do in allograft rejection. In the former situation the allogeneic stimulus is presumably very potent as the injected T cells interact with the highly immunogenic dendritic cells in the T areas of the host's secondary lymphoid tissue (Gowans, 1962; Steinman & Nussenzweig, 1980). In contrast, in the skin graft experiments only the grafted tissue contains the allogeneic stimulus.

In comparing the data reported here with results of experiments in the mouse (Sprent & Schaefer, 1986; Rosenberg *et al.*, 1986; Sprent & Schaefer, 1985; Sprent *et al.*, 1986) several differences are apparent. First, with experiments using media containing FCS it seems that mouse CD8+ T cells respond more vigorously than the corresponding rat T-cell subset. This difference arises, at least in part, because rat CD8+ T cells seem to mediate an autoregulatory activity. Second, unlike mouse cytotoxic T cells, rat CD8+ T cells show poor autonomous activity in their ability to reject skin allografts. This latter difference may be related to the strong inhibitory activity that rat serum has for T-cell activation, but there are reports of inhibitions of T-cell activation in mouse serum (Lelchuk & Playfair, 1985; Hardt *et al.*, 1981; Nelson & Schneider, 1974). It has been shown that, in the mouse, the ability of CD8+ T cells to mediate skin allograft rejection shows strong immune response gene effects with grafts in some genetic incompatibilities enjoying prolonged survival (Rosenberg *et al.*, 1986), while those in others are vigorously rejected. The rat and mouse data can be reconciled if one assumes that for the CD8+ T-cell subset the majority of the donor/recipient strain combinations examined in the rat are of the low responder type. This explanation is not supported by the *in vitro* data, however, which show that a readily detectable degree of alloreactivity can be demonstrated at least in FCS, for strain combinations in which skin allograft rejection *in vivo* occurs slowly or not at all when CD8+ T cells are used as the effector population.

The present experiments and those published on other studies in rats and mice (Salomon *et al.*, 1984; Brideau *et al.*, 1980; Cantor *et al.*, 1976) suggest that the CD4+ and CD8+ T-cell subsets are involved in a bilateral interaction with the CD4+ subset providing inducer activity, as IL-2, for the CD8+ subset, while the latter plays a predominately inhibitory role that affects both T-cell subsets. This negative feedback loop appears to be complicated by the fact that the CD8+ subset is, at early times after activation, capable of generating a positive autonomous response that is readily detectable in the mouse and that is present, though generally less obviously so, in the rat. It may be that rats and mice differ in the degree to which the positive and negative activities of CD8+ T cells are expressed and that in rats, but not in mice, the auto-suppressive activity of CD8+ T cells predominates. Further exploration of this point in the rat would be facilitated by a monoclonal antibody, like the one available in man, that distinguishes the CD8+ T cells that are responsible for auto-suppression from those that are precursors of cytotoxic T cells (Damle *et al.*, 1983).

In addition the cellular requirements for the suppressive activity of rat CD8+ cells need to be defined. Our data do not exclude the possibility that the CD8+ cells mediate their action indirectly, via an effect on macrophages (Oehler *et al.*, 1977), and this possibility is being explored currently.

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