Detection of *Salmonella*-specific L3T4$^+$ and Lyt-2$^+$ T cells which can proliferate *in vitro* and mediate delayed-type hypersensitivity reactivity

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SUMMARY

This study was based on an initial observation that, although culture of T cells from *Salmonella*-infected mice with concanavalin A induced both L3T4$^+$ T cells and Lyt-2$^+$ T cells to proliferate, there was a relative increase in the responsiveness of the Lyt-2$^+$ T cells in suspensions harvested from mice with secondary infection. Accordingly, primed T cells, obtained from the peritoneal cavities and spleens of mice that had received one or two intraperitoneal doses of *Salmonella* were examined for the presence of antigen-specific, class I major histocompatibility complex (MHC)-restricted Lyt-2$^+$ T cells. After primary infection with avirulent *Salmonella enteritidis* 11RX (11RX) only L3T4$^+$ T cells could be induced to proliferate in response to formalin-killed 11RX organisms, and a second dose of live 11RX did not change the phenotype of the responding T-cell population. In contrast, secondary challenge with *S. typhimurium* C5 (C5) generated cell populations where both L3T4$^+$ and Lyt-2$^+$ T cells proliferated when cultured with formalin-killed 11RX. Transfer of delayed-type hypersensitivity (DTH) using mixtures of primed T cells and either killed or live *Salmonella* organisms demonstrated that DTH was mediated by L3T4$^+$ T cells, and secondary infection with either the 11RX or C5 strain did not change this result. However, antigen-specific Lyt-2$^+$ T cells which mediated DTH reactivity were detected using a *Salmonella*-infected cell line which expressed MHC-coded class I but not class II products. These Lyt-2$^+$ T cells were present in the spleen and peritoneal cavity after secondary infection and in the peritoneal cavity late after a primary infection with 11RX.

INTRODUCTION

Immunity to intracellular bacterial parasites like *Listeria*, *Mycobacteria* and *Salmonella* has been shown to require antigen-specific T cells.[1-3] Until recently it was assumed that these T cells belonged to the L3T4$^+$ subset, because release of cytokines which activate macrophages that control proliferation of these organisms had been attributed to this T-cell subset[4,5] and because it was believed that expression of the L3T4 (CD4) or Lyt-2 (CD8) marker defined the functional role of T cells.[6] However, it is now certain that both subsets contain T

cells with helper and effector functions and that the major histocompatibility complex (MHC) molecules involved in antigen presentation determine the phenotype of the T cells induced to respond. L3T4$^+$ (CD4$^+$) T cells are stimulated by antigens in association with class II MHC products (and are said to be class II restricted), while antigens presented in the context of class I MHC molecules stimulate class I-restricted Lyt-2$^+$ (CD8$^+$) T cells.[7] It has been concluded that the pathway used for antigen processing determines whether antigenic determinants will associate with class I or class II MHC molecules.[8]

In this context, it is interesting to note that more recent studies in mice, including those using T-cell clones, have shown that both L3T4$^+$ and Lyt-2$^+$ T-cell subsets are required for effective protection against *Listeria* infection.[1,9] Evidently the L3T4$^+$ and Lyt-2$^+$ T cells co-operate to defend infected hosts against this bacterium, with the Lyt-2$^+$ T cells being ultimately responsible for bacterial clearance, especially during secondary infection.[9-11] Indeed, it has been suggested that co-operation between L3T4$^+$ and Lyt-2$^+$ T cells may only be observed when expansion of Lyt-2$^+$ T-cell clones is required, because they are present in low numbers.[9] Similarly, specific L3T4$^+$ T cells[12,13] and Lyt-2$^+$ T cells[14,15] have been detected in animals infected with *Mycobacteria*, although direct involvement of both subsets

in the elimination of infection has not been demonstrated. A number of groups have also reported that L3T4^{+} T cells from mice immunized with various strains of *Salmonella* respond to antigenic extracts of these organisms *in vitro* and *in vivo*, but the induction of antigen-specific Lyt-2^{+} T cells has not been reported,[16-19] although two studies using recombinant strains of *Salmonella* expressing unrelated antigens suggest that this might occur. Thus Lyt-2^{+} T cells specific for a circumsporozoite of *Plasmodium berghei*,[20] or ovalbumin[21] were induced in mice immunized with *S. typhimurium* expressing these antigens. However, a recombinant strain of *S. typhimurium* expressing the nucleoprotein of influenza A induced only CD4^{+} virus-specific T cells.[22]

Recent work has implicated T cells of the L3T4^{+} phenotype in the protection against *Salmonella* infection.[23] The ability of mice to clear *Salmonella* infection could be abolished by depleting them of L3T4^{+} T cells using anti-L3T4 monoclonal antibodies, and the ability of primed T cells to adoptively transfer resistance to *Salmonella* could be abrogated by *in vitro* pretreatment using anti-L3T4 and complement. However, these studies do not eliminate the possibility that T cells of the Lyt-2^{+} phenotype are also induced and play a significant role in immunity. Accordingly, the studies presented here were carried out to determine whether antigen-specific, class I MHC-restricted Lyt-2^{+} T cells are induced following infection with *Salmonella*. Because secondary infection with *Listeria* enhances the activity of Lyt-2^{+} T cells specific for this organism,[9,11] T-cell populations harvested from mice following secondary infection with *Salmonella*, as well as those obtained after primary infection, were examined.

MATERIALS AND METHODS

Animals
Sex-matched, 8-10-week-old (BALB/c × C57BL/6) F_1 (F_1) and C57BL/6 mice were used. Semi-lop-eared rabbits provided normal rabbit serum which was used as a source of complement.[19] All animals were obtained from the Central Animal House of The University of Adelaide, Australia.

Bacteria and bacterial antigens
Log-phase cultures of *S. enteritidis* 11RX (11RX; LD_{50} in normal mice of 2×10^6 organisms) and *S. typhimurium* C5 (C5; LD_{50} in normal mice of one to five organisms) were prepared as described previously.[19] For priming, F_1 mice were injected intraperitoneally (i.p.) with 10^5 live 11RX organisms per mouse; 3×10^4 live C5 or 8×10^6 live 11RX organisms per mouse was used as a secondary dose 3 or 6 weeks after the primary challenge, respectively. When used as the eliciting antigen in the delayed-type hypersensitivity (DTH) experiments, 11RX or C5 organisms were diluted to the appropriate concentration in antibiotic-free tissue culture medium and injected subcutaneously (s.c.) into the left hind footpad in the presence and absence of immune T cells. To prepare C5 organisms for invasion experiments, 1-ml aliquots of log-phase C5 suspension were pelleted in an Eppendorf centrifuge and resuspended to 5×10^8 bacteria/ml in antibiotic-free tissue culture medium. Formalin-killed 11RX organisms (F11RX) were prepared and stored as previously described.[19]

Tissue culture media
Hanks' balanced salt solution (HBSS; with 100 U/ml penicillin and 100 μg/ml streptomycin) was used to harvest and prepare all cell suspensions. RPMI-1640 (Gibco, Grand Island, NY) containing L-glutamine (2 mmol/l), β-mercaptoethanol (0·1 mmol/l), indomethacin (5 μg/ml), heat-inactivated fetal calf serum (FCS; 10% v/v; Flow Laboratories, Sydney, Australia) and the same antibiotics as those added to HBSS, was used for all tissue culture work (and will be referred to as culture medium). Culture medium without these antibiotics was used to prepare cell suspensions for DTH transfer experiments when live organisms were used as antigens. For *in vitro* culture of cells invaded with live *Salmonella*, culture medium was supplemented with gentamycin (40 μg/ml; Garamycin; Faulding Pty Ltd, Adelaide, South Australia) instead of penicillin and streptomycin. Indomethacin was added routinely to eliminate any variable inhibitory effects of secreted prostaglandins on T-cell activation because we planned to culture peritoneal cell populations in which the levels of macrophage activation (and prostaglandin production) might be expected to differ considerably.[24]

Preparation of lymphoid cell suspensions
Peritoneal cell (PC) and spleen cell (SC) suspensions were harvested from mice at various times after primary or secondary challenge with *Salmonella*, as described elsewhere.[25] Nylon wool (NW) non-adherent subpopulations of the PC and SC, containing mainly T cells, were prepared following a procedure established previously.[25] These cell suspensions were sometimes further purified (for use in DTH assays) by treating them with antibodies to the Ia[d] class II MHC product[26] and complement (1:10 and 1:20 final dilutions, respectively) using the one-step treatment method described earlier.[19] To determine the phenotype(s) of the cells mediating DTH reactivity, NW non-adherent PC and SC were treated with T-cell and T-cell subset-specific monoclonal antibodies (anti-Thy-1.2, anti-L3T4 and anti-Lyt-2.2) and complement (at a final dilution of 1:10 and 1:20, respectively), prior to local transfer to normal mice, together with and without antigen.[19]

Monoclonal antibodies and cell lines
30-H12 (anti-Thy-1.2), GK1.5 (anti-L3T4) and HO-2.2 (anti-Lyt-2.2) were generously donated by Dr L. Dent (Flinders Medical Centre, Adelaide, Australia; now of the Department of Microbiology and Immunology, The University of Adelaide, Australia) and MK-D6 (anti-Ia[d]) was purchased from ATCC (Rockville, MD). The murine mastocytoma cell line, P815, which expresses class I MHC products of the H-2[d] haplotype, was obtained from Dr K. Lafferty (Australian National University, Canberra, Australia) and maintained by continuous culture *in vitro* in 25 cm[3] and 75 cm[3] tissue culture flasks (Corning 25100-25 and 25110-75; Corning, NY) in culture medium and a gas phase of 5% CO_2 in air.

In vitro *proliferation assays*
These assays were set up in quadruplicate in 96-well flat-bottomed trays (Falcon 3072; Falcon, Lincoln Park, NJ) using 10^5 NW fractionated cells in 200-μl volumes of culture medium alone or culture medium containing F11RX or concanavalin A (Con A; 1 μg/ml, final concentration). The cultures were incubated for 3 days at 37° in an atmosphere of 10% CO_2, 7% O_2

and 83% N_2, before being pulsed with tritiated thymidine ([3H]thymidine; 37 kBq/well; Amersham Australia Pty. Ltd, Sydney, Australia) during the final 4 hr of incubation. Data are presented as the c.p.m. (mean ± SEM) or [3H]thymidine incorporated by each quadruplicate set of cultures. To provide sufficient cells for an analysis of the phenotype(s) of the proliferating cells, 24 replicate cultures of immune T cells were cultured with antigen or mitogen for 3 days in the standard way before treating them with antibodies and complement and pulsing with [3H]thymidine in the usual manner.

In vitro *invasion of P815 cells*

Various techniques of infecting P815 with live *Salmonella* were used before the procedure described here was adopted as the standard one. Rapidly dividing suspensions of P815 tumour cells were adjusted to 10^6 cells per ml in antibiotic-free culture medium and mixtures of P815 cells and bacteria were prepared in 10-ml sterile centrifuge tubes (Disposable Products, Laboratory Supply, Adelaide, South Australia) by mixing 1-ml volumes of the tumour cells with 200 μl of the same culture medium containing 10^8 C5 organisms. The mixtures of cells and bacteria were centrifuged for 10 min at 1500 *g*, incubated for 30 min in a 37° waterbath, resuspended and like suspensions pooled. The cells were washed thoroughly in antibiotic-free culture medium to remove any contaminating extracellular bacteria, resuspended to approximately 10^6 cells/ml in culture medium supplemented with gentamycin (40 μg/ml) and cultured overnight in 25 cm³ tissue culture flasks (Corning 25100-25) at 37° in an atmosphere of 5% CO_2 in air. After overnight culture, the tumour cells were washed, counted and adjusted to the required concentration in antibiotic-free culture medium.

Transfer of DTH reactivity, using live or killed organisms as eliciting antigen

Nylon wool fractionated PC and SC obtained from mice at various times following primary or primary and secondary immunization with *Salmonella* were mixed with antigen so that 50 μl of culture medium contained 10^6 cells and 2·5 μg F11RX, 10^5 live 11RX or 5 × 10^3 live C5. Groups of three normal F_1 mice were injected with 50 μl of each cell–antigen 'mix' in the left hind footpads. Control suspensions of cells alone and antigen alone were also injected into groups of three normal F_1 mice. The size of both the right and left hind footpads of all these groups of mice was measured 24 and 48 hr later to the nearest 0·05 mm, using Micrometer dial guage calipers (Type 130/3; Mercer, St Albans, U.K.). Results are expressed as the percentage increase in footpad size (mean ± SEM) for each group, calculated with respect to the size of the right, uninjected footpads.[16]

Transfer of DTH reactivity using C5-infected P815 cells as antigen

Mixtures of 10^6 purified T cells prepared from PC and SC of immunized mice and 10^4 C5-infected P815 cells were injected in 50-μl volumes into the left hind footpads of groups of three or four normal C57BL/6 mice and their hind footpads were measured 24 and 48 hr later. Control groups of three normal C57BL/6 mice, injected with immune T cells or C5-infected P815 alone, were also included in these assays. The data provided show the percentage footpad swelling (mean ± SEM) for each group of mice. The standard method of pretreatment with monoclonal antibodies and complement was used to determine the phenotype(s) of the cells responsible for the DTH reactivity detected using both assay systems. The standard dose of C5-infected P815 used in these assays was determined by injecting a range of doses of the infected cells into groups of three mice and selecting the minimum dose required to induce a significant response in the presence of immune T cells and a negligible amount of swelling when injected alone or with normal T cells.

RESULTS

In vitro proliferative responses

Preliminary data indicated that secondary challenge with either 11RX or C5 enhanced the yield of cells which could be removed from the peritoneal cavity of these mice. This effect correlated with the persistence of live bacteria—organisms of the much more virulent strain C5 persisted longer and induced a larger peritoneal cell exudate than 11RX (data not shown). To assess whether challenge with live *Salmonella* had induced a shift in the phenotypes of the T cells present at the original infection site and/or at a more distant site, a comparison was made of the ability of NW non-adherent populations of PC and SC obtained from 11RX-infected mice and 11RX-infected mice which were given a second dose of live 11RX 6 weeks after the primary 11RX dose, or a second dose of C5 after only 3 weeks, to respond to F11RX (1 μg/ml) and the T-cell mitogen Con A (1 μg/ml). The data (Tables 1 and 2) provided the basis for the further studies presented here. When stimulated by antigen, L3T4+ T cells were responsible for most of the proliferation induced in cultures of PC obtained from mice after a single or two doses of 11RX, whilst both L3T4+ T cells and Lyt-2+ T cells were induced to proliferate when PC harvested after secondary challenge with C5 were used (Table 1). Proliferation was not due to the recovery of persisting live *Salmonella* organisms with the PC, as cultures with no added antigen did not respond (data not shown). The responses induced by Con A were consistent with these results; both L3T4+ T cells and Lyt-2+ T cells were induced to proliferate, and there was a consistent reduction in the proportion of responding L3T4+ T cells in cultures of PC harvested from mice following secondary infection with C5 (Table 1). The specificity of the responses induced with 11RX antigen was confirmed by the repeated demonstration that NW fractionated T cells from uninfected mice did not respond to killed 11RX (but did respond to Con A) and the inability of any of the NW fractionated T-cell populations from infected mice to respond to a purified preparation of the antigenically unrelated K99 fimbrial protein of *Escherichia coli* (data not shown).

The results obtained using NW fractionated SC were not so clear cut. Despite their consistent ability to proliferate in response to Con A, the response of fractionated SC populations to antigen was quite variable, even when normal PC were added to ensure that lack of antigen-presenting cells (APC) was not limiting the response. In a set of 12 experiments, little or no response was observed on six occasions, responses which were fairly similar to those obtained with PC were obtained twice, and results similar to those presented in Table 2 were obtained on three separate occasions. Whenever proliferation was induced, only the cultures prepared from cells harvested after secondary infection with *either* 11RX *or* C5 contained proliferating T cells of both the L3T4+ and Lyt-2+ phenotypes.

Table 1. Characterization of the *in vitro* proliferative responses of primed T cells obtained from the peritoneal cavities of mice with primary or secondary *Salmonella* infections

Stimulus	Treatment†	[³H]thymidine incorporated* by NW fractionated immune PC after 3 days of culture with F11RX or Con A (1 μg/ml)		
		Schedule of immunization of PC donor		
		10^5 11RX	10^5 11RX + 8 × 10^6 11RX	10^5 11RX + 3 × 10^4 C5
F11RX	None	162,025 ± 3098	123,908 ± 1336	265,452 ± 9440
	Complement alone	158,495 ± 1567	131,435 ± 8050	252,973 ± 2920
	Anti-Thy-1.2 + complement	8062 ± 108	12,677 ± 1240	32,173 ± 2410
	Anti-L3T4 + complement	28,843 ± 2062	54,083 ± 5400	76,973 ± 2109
	Anti-Lyt-2.2 + complement	159,927 ± 5821	129,341 ± 2008	173,889 ± 14,784
	Anti-L3T4, anti-Lyt-2.2 + complement	26,901 ± 524	37,000 ± 478	42,130 ± 3772
Con A	None	186,465 ± 3201	175,039 ± 14,816	212,612 ± 5301
	Complement alone	196,002 ± 5342	141,228 ± 3686	192,747 ± 5919
	Anti-Thy-1.2 + complement	6032 ± 201	15,809 ± 1591	24,541 ± 2951
	Anti-L3T4 + complement	58,092 ± 3566	52,566 ± 2788	139,847 ± 3573
	Anti-Lyt-2.2 + complement	102,899 ± 3998	108,573 ± 3937	132,637 ± 3108
	Anti-L3T4, anti-Lyt-2.2 + complement	29,856 ± 209	35,015 ± 2081	49,293 ± 4315

* C.p.m. (mean ± SEM); the responses of the three types of cultures of untreated cells without stimulus were 444 ± 91, 1673 ± 899 and 2717 ± 1354 c.p.m. (sequence as above).

† Cells treated with the monoclonal antibodies and complement just prior to pulsing with [³H]thymidine.

Table 2. Characterization of the *in vitro* proliferative responses of primed T cells obtained from the spleens of mice with primary or secondary *Salmonella* infections

Stimulus	Treatment†	[³H]thymidine incorporated* by NW fractionated immune SC after 3 days of culture with F11RX or Con A (1 μg/ml)		
		Schedule of immunization of SC donor		
		10^5 11RX	10^5 11RX + 8 × 10^6 11RX	10^5 11RX + 3 × 10^4 C5
F11RX	None	52,453 ± 2068	109,638 ± 6976	42,758 ± 3204
	Complement alone	47,345 ± 7602	90,639 ± 3164	40,193 ± 3735
	Anti-Thy-1.2 + complement	14,566 ± 902	37,998 ± 557	14,974 ± 1176
	Anti-L3T4 + complement	28,086 ± 1023	58,071 ± 5957	24,360 ± 5221
	Anti-Lyt-2.2 + complement	48,477 ± 5872	70,983 ± 2096	28,886 ± 2007
	Anti-L3T4, anti-Lyt-2.2 + complement	26,445 ± 2295	40,127 ± 1959	15,701 ± 1528
Con A	None	285,132 ± 9864	324,113 ± 14,238	242,298 ± 3978
	Complement alone	278,997 ± 5488	255,760 ± 5443	255,228 ± 6805
	Anti-Thy-1.2 + complement	18,743 ± 1008	27,247 ± 1864	21,365 ± 1568
	Anti-L3T4 + complement	87,254 ± 2033	73,977 ± 13,658	186,300 ± 5215
	Anti-Lyt-2.2 + complement	134,223 ± 6722	159,006 ± 4713	149,605 ± 1236
	Anti-L3T4, anti-Lyt-2.2 + complement	29,322 ± 1099	32,482 ± 2537	31,603 ± 1918

* C.p.m. (mean ± SEM); the responses of the three types of cultures of untreated cells without stimulus were 891 ± 103, 1673 ± 899 and 704 ± 179 c.p.m. (sequence as above).

† Cells were treated with the monoclonal antibodies and complement just prior to pulsing with [³H]thymidine.
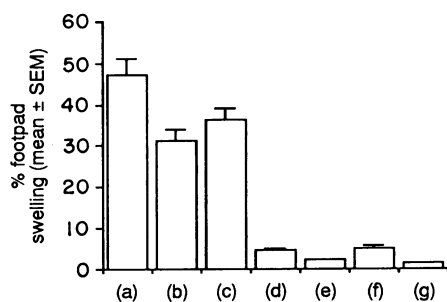
Figure 1. Local transfer of DTH. Left hind footpad swelling of normal F₁ mice injected with mixtures of NW fractionated PC harvested from mice 21 days after injection of 10^5 11RX and antigen, antigen alone or cells alone. The mixtures used were: (a) PC + killed 11RX; (b) PC + live 11RX; (c) PC + live C5; (d) killed 11RX; (e) live 11RX; (f) live C5; (g) PC. The thickness of both hind footpads of each mouse was measured at 24 hr and the data are presented as the percentage footpad swelling (mean ± SEM) for each group of three mice.

The implication of the consistent finding that treatment of cultured PC and SC with anti-L3T4+ plus anti-Lyt-2.2+ monoclonal antibodies and complement prior to pulsing with [³H]thymidine was unable to remove all the proliferating activity induced will be discussed later.

Local transfer of DTH reactivity using PC from *Salmonella*-immunized mice

We had shown previously that i.p. or i.v. infection of mice with 11RX induced PC and SC populations which could transfer DTH reactivity to normal mice, that PC were more effective than SC and that sensitization persisted for at least 70 days after infection and was mediated by non-adherent, Lyt-1+ T cells when a soluble antigen extract of 11RX was used to elicit DTH.[16] Because it is now clear that this form of 11RX antigen is likely to enter the antigen processing pathway which results in the formation of antigen–class II MHC complexes, we compared the ability of live and killed *Salmonella* organisms to elicit DTH. This approach was based on reports that both L3T4+ (class II-restricted) and Lyt-2+ (class I-restricted) T-cell mediating DTH responses to micro-organisms could be detected using live bacteria, whereas killed organisms elicited a response from class II-restricted T cells only.[27,28] Peritoneal cells harvested from mice 21 days after i.p. infection with 11RX were used to prepare immune T cells, and both C5 and 11RX organisms were used as 'live' antigen because C5 is more resistant to killing and persists in the tissues longer than 11RX[2] and, presumably, should provide a longer lasting source of antigen in a form favouring the detection of antigen-specific Lyt-2+ T cells. Preliminary experiments defined the minimum doses of 11RX and C5 which could be used to elicit a significant response in the presence of sensitized T cells without inducing significant footpad swelling in normal mice and established that measurements had to be made no later than 24–30 hr after cell transfer. Forty-eight hours after transfer, the amount of swelling induced using live organisms plus immune PC was comparable to that of controls injected with bacteria alone and could be attributed to bacterial multiplication because the swelling observed at 48 hr when killed 11RX was used to elicit DTH was always less than 10% for test animals and less than 5% for controls (data not shown). The experiment was carried out on four separate occasions, yielding very similar results. Figure 1 shows the data
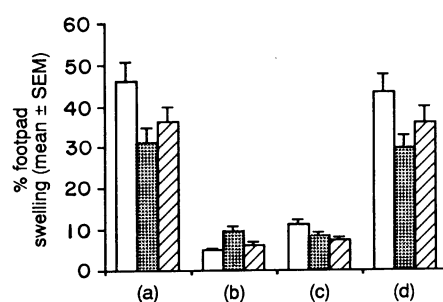

Figure 2. Characterization of the DTH effector cells. NW fractionated cells (PC) harvested from the peritoneal cavity 21 days after injection of 11RX were treated with (a) complement; (b) anti-Thy-1.2 + complement; (c) anti-L3T4 + complement; or (d) anti-Lyt-2.2 + complement, prior to transfer to the left hind footpad of normal F₁ mice together with either F11RX (unshaded columns), live 11RX (shaded columns) or live C5 (hatched columns). The thickness of both hind footpads of each mouse was measured at 24 hr and data are presented as the percentage footpad swelling (mean ± SEM) for each group of three mice.

from one of these experiments and indicates that all three antigens elicited significant swelling in the presence of 10^6 NW fractionated PC from immunized animals. Pretreatment of the immune T cells with various monoclonal antibodies and complement showed that the cells responsible for transfer of DTH responses were L3T4+ T cells in each case; data representative of a series of four such experiments are presented in Fig. 2.

Because *in vitro* proliferation assays detected a greater response by Lyt-2+ T cells in PC suspensions obtained from mice following secondary *Salmonella* infection, the above experiments were repeated using T cells prepared from PC and SC of mice at various times after secondary challenge with either 11RX or C5. The results obtained confirmed the data presented in Fig. 1. The levels of DTH transferred with immune T cells prepared from these PC were very similar to those obtained using PC from mice given only one dose of 11RX, and only MHC class II-restricted T cells were detected again, even when live organisms were used to elicit the response (data not shown). The responses transferred with immune T cells prepared from SC were significantly lower, with 20% swelling the maximum ever detected, and were also mediated by T cells of L3T4+ phenotype (data not shown). Two obvious explanations for these results were that no *Salmonella*-specific Lyt-2+ T cells able to mediate DTH had been induced or that such cells could not be detected because of preferential presentation of *Salmonella* antigens in association with class II MHC products under the conditions used in the experiments. The following experiments provided evidence to support the second alternative.

Detection of antigen-specific Lyt-2+ DTH effector T cells after secondary immunization with *Salmonella*

Numerous attempts to antigen-pulse various cells with lysates of 11RX and C5 were uniformly unsuccessful in obtaining antigen presentation with MHC class I products. Presentation of *Salmonella* antigens in association with class I MHC products was achieved by using the MHC class I+, class II− tumour cell line P815 (H-2ᵈ haplotype) and infecting it with C5. The method of infection used did not affect the viability of P815 cells, even after overnight incubation. To verify that the bacteria had entered the tumour cells, stained smears of these cells were
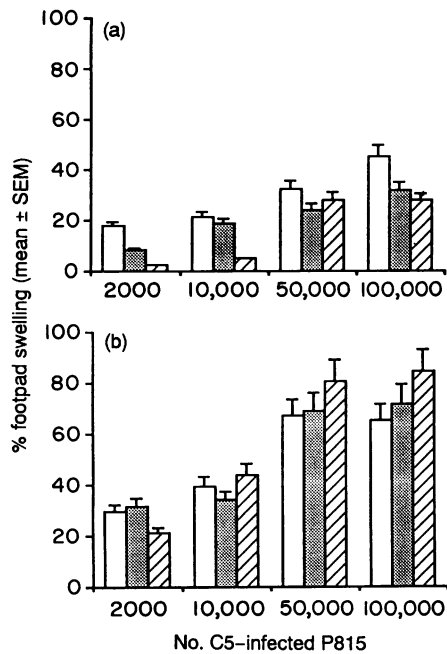
Figure 3. Ability of C5-infected P815 cells to elicit DTH. The swelling induced in left hind footpads of normal C57BL/6 mice (a) 24 and (b) 48 hr after transfer of mixtures of NW fractionated and anti-Ia^d+complement-treated PC (unshaded columns) or SC (shaded columns) harvested from mice with a secondary infection of C5 and various doses of C5-infected P815 is shown. Control suspensions of C5-infected P815 in culture medium were also included (hatched columns) and the thickness of both hind footpads was measured 3, 12 and 24 and 48 hr later. Data obtained at 24 and 48 hr are presented as the percentage footpad swelling (mean $\pm$ SEM) for each group of three mice.

examined using oil immersion microscopy. Bacteria were seen within vacuoles or were free in the cytoplasm, although the number of bacteria associated with P815 cells varied considerably and ranged from none to as many as 20 per cell. There were few if any bacteria outside the cells and none 'stuck' around the edges of the cells. The recovery of live C5 from infected P815
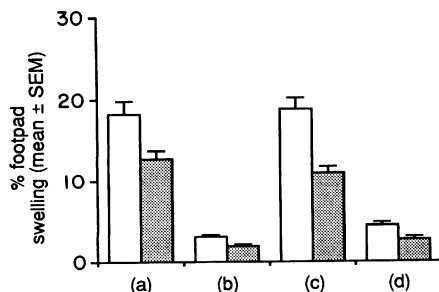

Figure 4. Characterization of the cells which mediate the DTH responses induced by C5-infected P815 cells. Purified, immune T cells obtained from the peritoneal cavities (unshaded columns) and spleens (shaded columns) of mice with a secondary C5 infection were treated with (a) medium; (b) anti Thy-1.2+complement; (c) anti-L3T4+complement; or (d) anti-Lyt-2.2+complement, prior to transfer to the left hind footpads of normal C57BL/6 mice together with 10^4 C5-infected P815. The thickness of both hind footpads was measured 3 and 24 h later and data representative of four experiments are presented as the percentage footpad swelling at 24 hr (mean $\pm$ SEM) for each group of three mice.

cells was determined in 10 separate experiments and was found to vary from 1–6 bacteria per tumour cell.

Preliminary cell transfer experiments, using immune T cells prepared from PC and SC of F$_1$ mice 14 days after secondary challenge with C5, established that 10^4 C5-infected P815 cells per mouse could be used to elicit DTH responses. The T cells used were prepared by NW fractionation, followed by treatment with anti-Ia^d and complement to reduce the possibility that APC other than the C5-infected P815 cells could be involved in antigen presentation. In addition, normal C57BL/6 mice (H-2^b haplotype) were chosen as recipients because the specificity of class II MHC-restricted 11RX-primed F$_1$ T cells is preferentially restricted to products of the H-2^d haplotype.[16,25] Twenty-four hour after transfer of 10^4 C5-infected P815 and 10^6 purified immune T cells, significant DTH responses were evident, with negligible swelling detected when C5-infected P815 or immune T cells were injected alone (Fig. 3a). Results obtained 48 hr after transfer illustrate a limitation of using live *Salmonella* as
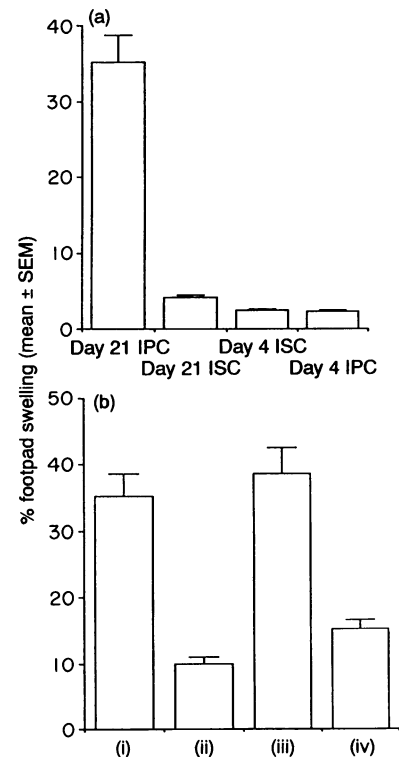

Figure 5. Detection and characterization of DTH effector cells after primary immunization with *Salmonella*. The thickness of both hind footpads of all groups of mice was measured 3 and 24 hr after cell transfer and data obtained at 24 hr and representative of three experiments of each type are presented as the percentage footpad swelling (mean $\pm$ SEM) for each group of three mice. (a) The left hind footpads of normal C57BL/6 mice were injected with mixtures of 10^6 purified, primed T cells harvested from the peritoneal cavities and spleens of mice immunized with 11RX 4 or 21 days earlier (day 4 IPC/ISC and day 21 IPC/ISC, respectively) and 10^4 C5-infected P815. (b) C5-infected P815 and 10^6 purified, immune T cells prepared from the day 21 PC suspension, treated with (i) medium; (ii) anti-Thy-1.2+complement; (iii) anti-L3T4+complement; (iv) or anti-Lyt-2.2+complement, were transferred to the left hind footpad of normal C57BL/6 mice and data obtained 24 hr later are presented as percentage footpad swelling at 24 hr (mean $\pm$ SEM) for each group of three mice.

antigen. The amount of footpad swelling had increased considerably, both in the presence and in the absence of T cells (Fig. 3b) and could be attributed to bacterial multiplication because uninfected P815 cells alone induced little or no swelling at both time-points (5% maximum, data not shown). The cells mediating DTH at 24 hr were shown to be T cells expressing the Lyt-2.2⁺, Thy-1.2⁺ markers (Fig. 4).

Detection of Lyt-2⁺ DTH effector T cells following primary immunization with 11RX

An identical approach was used to demonstrate the presence of class I-restricted Salmonella-specific DTH effector T cells in cell populations harvested from mice after only one dose of 11RX. T cells prepared using PC and SC harvested 4 or 21 days after infection were tested and it was found that only the PC harvested from mice 21 days after infection contained cells able to transfer DTH reactivity (Fig. 5a), and pretreatment of these cells with various monoclonal antibodies and complement revealed that they belonged to the Lyt-2⁺ phenotype of T cells (Fig. 5b). Thus, Lyt-2⁺ T cells capable of mediating DTH appeared late after initial injection of 11RX, and could be detected only in PC suspensions.

DISCUSSION

Infection of F₁ mice with Salmonella induced both L3T4⁺ and Lyt-2⁺ T cells, with an increased involvement of class I-restricted Lyt-2⁺ T cells during secondary challenge, particularly when the more virulent C5 organisms were used. The results obtained are consistent with the work of others who have demonstrated that antigen-specific L3T4⁺ and Lyt-2⁺ T cells are induced following infection with Listeria[1,11,29] and Mycobacteria[12-14] and that secondary infection enhanced the activity of antigen-specific Lyt-2⁺ T cells, and provide a basis for further work designed to define the role of these T-cell subsets in immunity to Salmonella. Unfortunately, they do not provide any insight which would explain the differences we observed between responding cell populations obtained from the spleen and the peritoneal cavity. In particular, reasons for the variation in responsiveness of NW fractionated SC of immunized mice to Salmonella antigens remain uncertain.

It is generally accepted that T cells of the L3T4⁺ phenotype are induced by antigens, including micro-organisms, that are taken up by APC and processed in the endosomal compartment before being presented in association with class II MHC molecules. It has also been established that Lyt-2⁺ T cells are induced by complexes of class I MHC molecules, and antigen fragments are formed when antigens enter a different (endogenous or cytoplasmic) processing pathway.[8] The induction of Lyt-2⁺ T cells in mice infected with Listeria can be explained by the production of listeriolysin O[30] which allows the organisms to enter the cytosol where they multiply[31] and, presumably, gain access to the class I presentation pathway. The report that complexes of Listeria antigens and class I MHC products were generated from only those L. monocytogenes organisms which expressed listeriolysin O provides support for this possibility.[32]

An alternative explanation is required for the induction of class I-restricted T cells following infection with Mycobacteria and Salmonella because no 'listeriolysin O-like' factor has been described for either organism. Obvious mechanisms which may

explain how antigenic determinants of these organisms associate with class I MHC molecules are that bacteria multiplying within phagolysosomes of infected macrophages 'break out' into the cytoplasm without lysing the cells and/or that some bacterial antigens 'leak' into the cytosol. A recent report has shown that small amounts of liposome-encapsulated antigen were released into the cytosol and were subsequently presented in the context of class I MHC products,[33] supporting the latter possibility. We have observed cytospin smears of C5-infected P815 cells which contain some cells with bacteria clearly inside vacuoles and others which appear to contain organisms 'free' in the cell cytoplasm (data not shown). Alternatively, exogenous small peptides of Mycobacteria and Salmonella may bind directly to class I MHC molecules on the surface of APC[34] or to class I MHC molecules as they recycle through endosomes.[35] Although there is evidence for such recycling only in T cells, it is possible that during infection with intracellular bacterial parasites the class I MHC molecules of macrophages (APC) may also recycle through endosomes and associate with exogenously derived antigenic peptides present in these compartments and subsequently induce class I-restricted Lyt-2⁺ T cells. Recent observations by others[21] and our observation that F11RX induced the proliferation of class I-restricted antigen-specific Lyt-2⁺ T cells when PC harvested from mice with a secondary C5 infection were used, are consistent with this possibility. Because F11RX is a form of antigen which is usually presented in association with class MHC products, it appears that secondary infection with C5 organisms modifies APC function, favouring presentation of antigens in association with class I MHC products, although further work is required to confirm these observations.

The demonstration that treatment with anti-Thy-1.2 and complement reduced the proliferative activity of NW fractionated immune PC cultured with antigen more than treatment with a mixture of anti-L3T4, anti-Lyt-2.2 and complement suggests that 'double negative' (L3T4⁻,Lyt-2⁻) T cells were also present in these cultures. Since the majority of peripheral double-negative T cells express antigen-binding receptors of the γδ type,[36] it seems possible that a subset of γδ T cells was induced to proliferate. This would be consistent with reports that a significant proportion of both human and murine γδ T cells recognize T-cell epitopes of heat-shock proteins expressed by a number of bacteria and eukaryotic cells.[37-39] In fact, S. typhimurium organisms within macrophages have been reported to increase their synthesis of the two heat-shock proteins GroEL and DnaK, shown to be immunodominant antigens for many infectious organisms.[40] Therefore, it might be expected that infection with Salmonella induced γδ T cells able to proliferate in vitro in response to antigen. More detailed characterization of these responses is required to confirm the presence of Salmonella-specific γδ T cells, but preliminary experiments carried out by another member of this laboratory using anti-γδ T-cell receptor antibodies and complement have indicated that such a population of γδ T cells is generated during Salmonella infection and can be induced to proliferate in vitro when stimulated with Con A (Bertram, unpublished data).

In addition to an increase in the proliferative activity of class I-restricted Lyt-2⁺ T cells following secondary Salmonella infection, class I-restricted DTH effector T-cell activity was also enhanced. However, unlike other systems where live organisms were used as antigen to detect class I-restricted DTH effector T

cells,[27,28] *Salmonella*-specific Lyt-2$^+$ T cells were only detected using a very stringent system, where antigen presentation was restricted to an association with class I MHC products. These data imply that fewer Lyt-2$^+$ T cells were induced by *Salmonella* infection, making them harder to detect than similar cells in *Listeria* and Sendai virus-infected mice, or that 'normal' APC present *in vivo* process most of the antigens of live *Salmonella* via the endosomal pathway, or both. In any case, examination of cytospin smears of C5-infected P815 established that the assay system used to detect class I-restricted Lyt-2$^+$ T cells was not ideal because the P815 cells were not uniformly infected. Some cells contained no bacteria and others up to 20 bacteria per cell (data not shown). Hence, a more effective method of antigen-pulsing is required to allow a more detailed study of the antigen-specific T cells induced by *Salmonella* infection. One obvious approach would be to 'load up' APC with well-defined peptides of *Salmonella* antigens, via the osmotic lysis of pinosomes,[41] to ensure that more cells present antigen in association with class I MHC molecules.

ACKNOWLEDGMENTS

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