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Induction of Lyt-2$^+$ cytotoxic T lymphocytes following primary and secondary *Salmonella* infection

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SUMMARY

Investigations of the cytotoxic activity of T cells induced following one or two intraperitoneal doses of live *Salmonella* revealed that cytotoxicity was restricted to the Lyt-2$^+$ T-cell subset and was enhanced following secondary infection with *Salmonella*. Initial studies using the lectin-dependent cellular cytotoxicity (LDCC) assay detected Lyt-2$^+$ cytotoxic T cells in peritoneal cell suspensions of *S. enteritidis* 11RX (11RX)-infected mice, with the peak of activity occurring 5 days after infection. This did not correlate with the proliferative activity of these cells, which peaked 10–12 days after infection. Secondary challenge with 11RX or *S. typhimurium* C5 (C5) induced a rapid increase in the cytotoxic activity of Lyt-2$^+$ peritoneal T cells and was detected even 21 days later. The antigen specificity of some of these cells was confirmed in cytotoxicity assays using P815 tumour cells infected with 11RX organisms as targets. No cytotoxic activity was detected in the spleen cell suspensions of infected (and normal) mice unless the cells were first activated by *in vitro* culture with concanavalin A (Con A). Both types of activated spleen cells showed LDCC but *Salmonella*-specific cytotoxic Lyt-2$^+$ T cells were detected only in spleen cell (SC) cultures prepared from mice challenged with a second dose of *Salmonella*.

INTRODUCTION

Effective clearance of the intracellular bacterial parasites *Listeria* and *Mycobacteria* from mouse tissues requires cell-mediated immune responses[1,2] involving both major histocompatibility complex (MHC) class I-restricted Lyt-2$^+$ T cells and class II-restricted L3T4$^+$ T cells,[3,4] although the clearance of *Listeria* infection appears to require only the Lyt-2$^+$ T-cell subset.[3,5,6] It is certain that immunity to *Salmonella* is also cell-mediated[7] and, although the T-cell subsets induced by *Salmonella* infection have not been well characterized, the clearance of a temperature-sensitive strain of *Salmonella* has been reported to require T cells expressing the L3T4$^+$ marker.[8] Consistent with this, we have shown that infection of mice with *Salmonella enteritidis* 11RX (11RX) induced *Salmonella*-specific L3T4$^+$ T cells able to proliferate and release cytokines when cultured *in vitro* with 11RX antigens and to transfer delayed-type hypersensitivity (DTH) to normal mice.[9,10] However, our recent studies have shown that Lyt-2$^+$ T cells with the same characteristics are also induced during infection with 11RX and that, like *Listeria*, secondary challenge with *Salmonella* enhanced the involvement of antigen-specific Lyt-2$^+$ T cells, particularly when the more virulent *S. typhimurium* C5 (C5) strain is used.[11] We believe that the lack of a satisfactory method for preparing antigen-pulsed cells which present *Salmonella* antigen fragments in association with class I MHC products has been a major obstacle in the detection of *Salmonella*-specific T cells of the Lyt-2$^+$ phenotype and analysis of their function. In our hands, the methods used to present *Listeria* antigens in association with class I MHC products on antigen presenting and target cells[12,13] could not be directly applied to *Salmonella*, and the successful approach that we have developed to detect *Salmonella* antigen-specific T effector cells of the Lyt-2$^+$ phenotype requires a great deal of time and effort and has obvious limitations.[11]

Measurement of the cytotoxic activity of T-cell populations is often used as an indication of activation of a cell-mediated (T-cell) immune response and can detect T cells of the Lyt-2$^+$ phenotype. For example, extensive characterization of the T cells induced by infection with *Listeria* has revealed that both

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Abbreviations: APC, antigen-presenting cell(s); C5, *S. typhimurium* C5; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; F_1 mice, (BALB/c × C57BL/6) F_1 mice; FCS, fetal calf serum; F11RX, formalin-killed 11RX; i.p., intraperitoneally; IPC, peritoneal cells from immunized mice; ISC, spleen cells from immunized mice; LDCC, lectin-dependent cellular cytotoxicity; MHC, major histocompatibility complex; NPC, peritoneal cells from normal mice; NW, nylon-wool; PC, peritoneal cells; 11RX, *Salmonella enteritidis* 11RX; SC, spleen cell(s).

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L3T4$^+$ and Lyt-2$^+$ antigen-specific cytotoxic T lymphocytes (CTL) are present.[12-14] The demonstrations that infection with recombinant strains of *S. typhimurium* resulted in the induction f Lyt-2$^+$ (CD8$^+$) CTL specific for circumsporozoites of *Plasmodium berghi*[15] and L3T4$^+$ CTL specific for the nucleoprotein of influenza A virus[16] provided the impetus for the present studies. At the beginning of the studies reported here, a method of preparing suitable *Salmonella* antigen-pulsed target cells had not been devised. Therefore, we decided to investigate the possibility of using the lectin-dependent cellular cytotoxicity (LDCC) assay to determine whether any CTL were induced during *Salmonella* infection. This approach was based on the demonstration that antigen-specific CTL have the potential to lyse not only target cells bearing the appropriate antigens, but also antigenically unrelated target cells, provided that they express syngeneic, allogeneic or xenogeneic MHC products and lectins such as phytohaemagglutinin or concanavalin A (Con A) are present.[17-19] Initial experiments using LDCC assays provided evidence that Lyt-2$^+$ CTL, but not L3T4$^+$ CTL, were induced during infection with *Salmonella*. These studies were later extended using P815 tumour cells infected with *Salmonella* as targets. The results obtained are presented here.

MATERIALS AND METHODS

Animals

Sex-matched, 8–10-week-old (BALB/c × C57BL/6) F_1 (F_1) mice were used as experimental animals and normal rabbit serum was used as a source of complement.[11]

Bacteria, tissue culture media, lymphoid cell suspensions and monoclonal antibodies

The two strains used were *S. enteritidis* 11RX (11RX) and *S. typhimurium* C5 (C5). Log-phase cultures of 11RX (LD_{50} in normal mice of 2×10^6 organisms) and C5 (LD_{50} in normal mice of 1–5 organisms) were prepared as described previously.[11] For primary infection, 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. The tissue culture media and the methods used to prepare nylon wool (NW) non-adherent peritoneal cells (PC) and spleen cells (SC) are described in the companion paper.[11] Details of the cell lines used to prepare monoclonal antibodies (mAb) and the method used to determine the phenotype(s) of cytotoxic cells can also be found there.

In vitro *activation of spleen cells with mitogen*

SC suspensions prepared from normal mice and mice 4 days after primary or secondary challenge with *Salmonella* were adjusted to 10^6 cells/ml in culture medium containing Con A (1 μg/ml), 1-ml aliquots dispensed into the wells of a 24-well flat-bottomed tray (Corning 25820; Corning, NY) and incubated at 37° for 3 days in an atmosphere of 10% CO_2, 7% O_2 and 83% N_2. On the third day the cultures were gently shaken (Titretek Tray Shaker; Flow Laboratories, Sydney, Australia) and the cells harvested and pooled using a sterile pasteur pipette. The number of viable cells was determined and the cells were adjusted to the required concentration(s) in culture medium.

Measurement of proliferation of peritoneal cells

PC were harvested from mice at various times after immunization with 11RX, fractionated on NW columns to remove inhibitory cells[10] and 200-μl aliquots of each suspension containing 5×10^5 cells in culture medium were dispensed into four replicate wells of a 96-well flat-bottomed tray and pulse-labelled with tritiated thymidine ([^{3}H]TdR; 37 kBq/well; Amersham Australia Pty. Ltd, Sydney, Australia) for 4 hr at 37°. The amount of proliferation was determined by measuring the [^{3}H]TdR incorporated during this period by each sample and results were expressed as c.p.m. (mean ± SEM) for each replicate set of cells.

In vitro *invasion of P815 cells with 11RX*

The method used for maintenance of these cells and the invasion procedure are described in the companion paper, where C5 instead of 11RX was used to infect P815.[11]

Labelling target cells with ^{51}Cr

Suspensions of normal P815 and 11RX-infected P815 were adjusted to 5×10^6 cells/ml and 500-μl aliquots were dispensed into separate sterile siliconized glass centrifuge tubes. Each cell suspension was incubated with 50 μCi $Na_2{}^{51}CrO_4$ (Amersham) for 1 hr at 37°, with shaking every 20 min before being washed in fresh prewarmed culture medium. The cells were resuspended in 5 ml of warm culture medium, underlaid with approximately 1·5 ml fetal calf serum (FCS) and centrifuged again at 250 ***g*** for 3 min. Each resultant pellet was resuspended in 5 ml of fresh culture medium and incubated for a further 1 hr at 37°. Finally, the cells were centrifuged through FCS again, counted and adjusted to 2×10^5 cells/ml in culture medium. When preparing Con A-treated target cells for the LDCC assays, Con A (0·5 ml, 100 μg/ml) was added to the 5-ml suspension of ^{51}Cr-labelled target cells for the last 20 min before the cells were centrifuged through FCS and adjusted to 2×10^5 cells/ml.

In vitro *cytotoxicity assays*

Effector cell suspensions were adjusted to 5×10^6, 10^6 and 2×10^5 cells/ml in culture medium and mixed with an equal volume of ^{51}Cr-labelled target cells (2×10^5/ml). Two hundred-microlitre aliquots of each effector cell–target cell mix were added to four wells of a 96-well round-bottomed tray (76-042-05, Linbro; ICN Biochemicals, Sydney, Australia) and incubated for 4 hr at 37° in an atmosphere of 5% CO_2. The amount of ^{51}Cr released was determined by sampling 100 μl of each culture supernatant and measuring the radioactivity present using a gamma counter (Packard Auto-Gamma 5650; Packard, IL). Control suspensions of culture medium containing ^{51}Cr-labelled target cells alone were also included to determine the amount of ^{51}Cr spontaneously released by the target cells in the absence of effector cells (spontaneous release c.p.m.). Four replicate 100-μl volumes of ^{51}Cr-labelled target cells (at 2×10^5/ml) were counted in the gamma counter to assess the total amount of radioactivity taken up by the cells (total c.p.m.). The percentage cytotoxicity of each effector cell population was calculated using the mean c.p.m. recorded for each replicate target cell–effector cell mix in the following equation:

$$\% \text{ cytotoxicity} = 100 \times \frac{\text{experimental release c.p.m.} - \text{spontaneous release c.p.m.}}{\text{total c.p.m.} - \text{spontaneous release c.p.m.}}$$

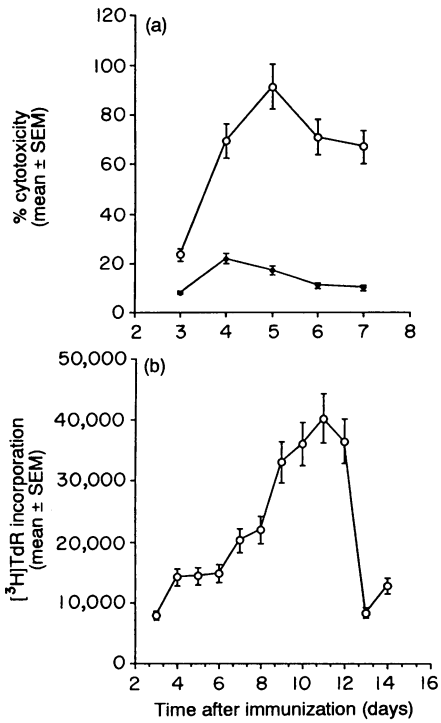

Figure 1. The cytotoxic and proliferative activity of PC from 11RX-infected mice. (a) NW-fractionated PC were prepared from cells harvested at various times after infection with 11RX and their cytotoxicity determined using the LDCC assay, using Con A-treated ^{51}Cr-labelled P815 (○) and untreated ^{51}Cr-labelled P815 (●) as target cells. Data obtained using a ratio of 25 effector cells for every target cell are presented as percentage cytotoxicity (mean ± SEM). (b) The proliferative activity of the NW-fractionated PC prepared from cells harvested at various times after infection with 11RX was measured using a 4-hr period of incubation at 37° with [^{3}H]TdR. Data are presented as the c.p.m. incorporated (mean ± SEM) of quadruplicate samples. [The c.p.m. (mean ± SEM) incorporated by NW-fractionated PC obtained 21 and 28 days after infection was 10,257 ± 865 and 6582 ± 218, respectively.]

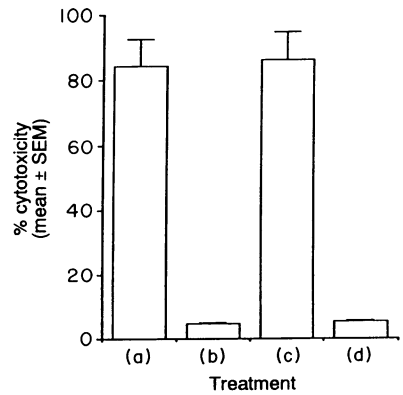

Figure 2. Phenotype of the cytotoxic cells detected using the LDCC assay. Prior to assay, NW-fractionated PC from mice injected with live 11RX 5 days earlier were treated with (a) complement; (b) anti-Thy-1.2 + complement; (c) anti-L3T4 + complement; or (d) anti-Lyt-2.2 + complement. The data obtained with an effector : target cell ratio of 25 : 1 are presented as percentage cytotoxicity (mean ± SEM) of quadruplicate samples.

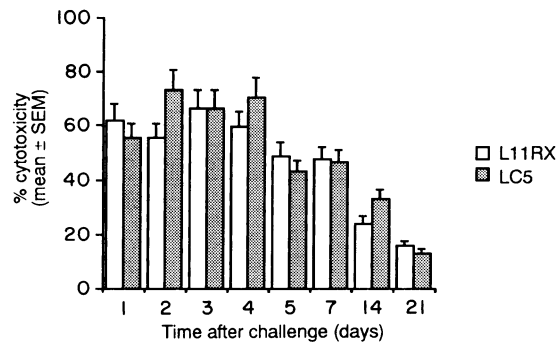

Figure 3. LDCC assay of cytotoxic activity of PC harvested from mice after secondary challenge with *Salmonella*. The PC tested were obtained from mice 1–21 days after secondary challenge with either live 11RX (L11RX) or live C5 (LC5) and were NW fractionated before being incubated with Con A-treated ^{51}Cr-labelled P815 as targets at an effector : target cell ratio of 25 : 1. The data are expressed as percentage cytotoxicity (mean ± SEM) of quadruplicate samples; cytotoxic activity against untreated ^{51}Cr-labelled P815 cells was never greater than 5% (not shown).

RESULTS

Cytotoxicity and proliferative activity of PC after primary immunization with 11RX

NW-fractionated, T-cell enriched PC harvested from mice at various times after i.p. infection with 11RX were incubated with Con A-treated target cells at a ratio of 25 : 1 and additional aliquots of these PC were used to determine their proliferative activity. The PC were cultured for 4 hr at 37° with [^{3}H]TdR before measuring the amount of radioactivity they had incorporated. A summary of the data obtained is presented in Fig. 1. Clearly, infection with *Salmonella* induced a cytotoxic population of cells, with maximum activity detected on the fifth day of infection (Fig. 1a), but which persisted at lower levels, in the range of 10–15%, throughout the period that 11RX organisms could be recovered from the peritoneal cavity (data not shown). No significant cytotoxic activity (5% maximum) was detected in PC suspensions obtained from normal mice or mice injected with killed 11RX organisms 3 days to 2 weeks before cell harvest (data not shown). The peak of cytotoxic activity correlated with the maximum recovery of viable organisms from these mice (data not shown) but did not coincide with the peak of proliferative activity of PC which occurred on day 11 of infection (Fig. 1b).

Since the LDCC assay detects both MHC class I- and class II-restricted T cells, the phenotype of the cytotoxic cells detected at the peak of the response was determined. NW-fractionated PC harvested from mice 5 days after infection were treated with various mAb and complement before being incubated with Con A-treated ^{51}Cr-labelled P815, and their cytotoxicity was assayed in the usual manner. The results shown in Fig. 2 are representative of five experiments which yielded very similar results, indicating that only Lyt-2$^+$ CTL were present in this population.

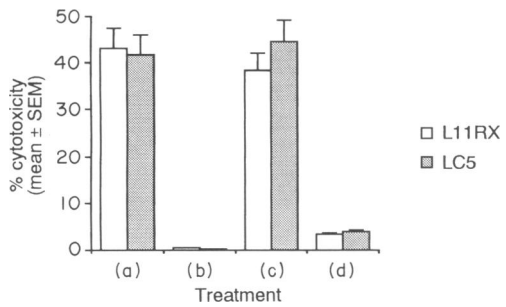

Figure 4. Phenotype of the cytotoxic cells present after secondary challenge with live 11RX (L11RX) or C5 (LC5), detected using the LDCC assay. NW-fractionated PC harvested 4 days after secondary infection were treated with (a) complement; (b) anti-Thy-1.2+complement; (c) anti-L3T4+complement; or (d) anti-Lyt-2.2+complement before incubating each effector population with Con A-treated ^{51}Cr-labelled P815 cells. The data obtained with an effector:target cell ratio of 25:1 are expressed as percentage cytotoxicity (mean±SEM) of quadruplicate samples.

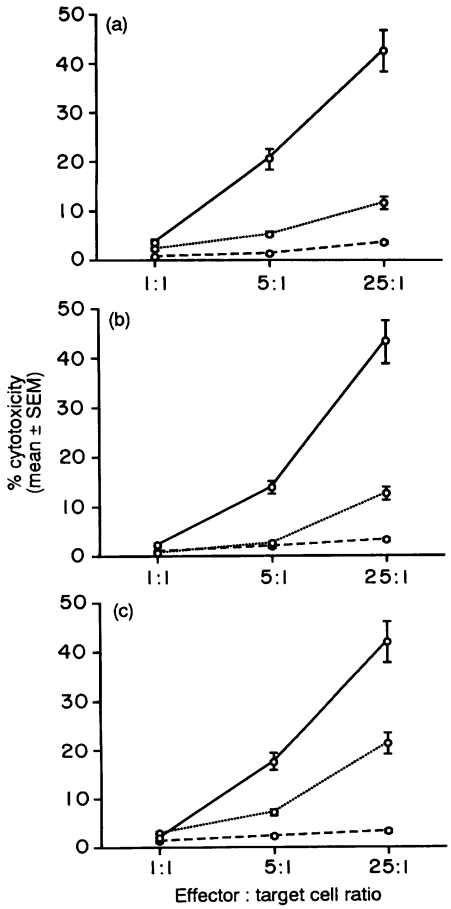

Figure 5. *Salmonella*-specific cytotoxic activity of PC from *Salmonella*-infected mice. NW-fractionated PC suspensions from mice infected with (a) 11RX 5 days earlier or given a secondary challenge with (b) 11RX or (c) C5 4 days earlier were incubated with Con A-treated ^{51}Cr-labelled P815 (O——O), ^{51}Cr-labelled P815 cells infected with 11RX (O·····O), or untreated ^{51}Cr-labelled P815 cells (O- - -O). The data obtained are expressed as percentage cytotoxicity (mean±SEM) of quadruplicate samples.

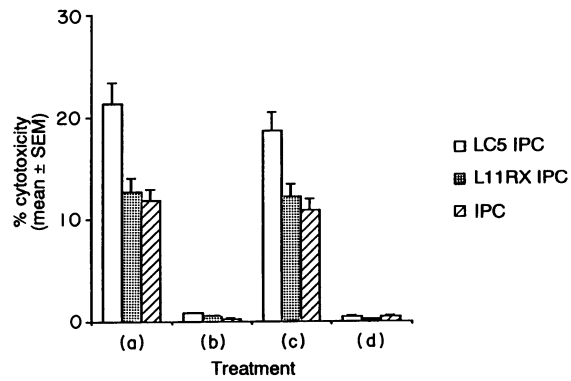

Figure 6. The phenotype of the *Salmonella*-specific CTL. NW-fractionated PC suspensions from mice given a secondary challenge of live C5 (LC5 IPC) or live 11RX (L11RX IPC) or one injection of live 11RX (IPC) were treated with (a) complement; (b) anti-Thy-1.2+complement; (c) anti-L3T4+complement; or (d) anti-Lyt-2.2+complement, prior to incubating them with the ^{51}Cr-labelled 11RX-infected P815 at a ratio of 25:1. The data obtained are expressed as percentage cytotoxicity (mean±SEM) of quadruplicate samples. Control suspensions of the various effector cells and ^{51}Cr-labelled uninfected P815 were also included; the maximum cytotoxicity detected was 1·2%.

Detection of Lyt-2$^+$ CTL following secondary *Salmonella* infection

Because parallel studies had indicated that secondary infection with *Salmonella* enhanced the levels of Lyt-2$^+$ T cells that could proliferate *in vitro* and transfer DTH to normal animals,[11] the cytotoxic activity of NW-fractionated PC harvested at various times after secondary challenge with either C5 or 11RX was compared in the LDCC assay using Con A-treated ^{51}Cr-labelled P815, and Fig. 3 presents data obtained, which were confirmed in several repeat experiments. They show that cytotoxic activity was induced very soon after secondary challenge and that considerable cytotoxic activity was maintained for up to 7 days after challenge, with lower levels thereafter. Killing of untreated ^{51}Cr-labelled P815 cells was minimal with all effector cell populations (approximately 3–5% maximum, data not shown). The cells responsible for this cytotoxic activity were also found to express the Lyt-2$^+$, Thy-1$^+$ phenotype; the results obtained using cells harvested 4 days after secondary challenge with 11RX or C5 are shown in Fig. 4.

Specificity of the Lyt-2$^+$ CTL in the PC populations

Since the LDCC assays indicated that *Salmonella* infection had induced Lyt-2$^+$ T cells, with no evidence of L3T4$^+$ CTL, further analysis of the CTL detected was carried out. Preliminary studies revealed that P815 infected with either C5 or 11RX were both lysed by immune T cells but more detailed analyses were carried out using only ^{51}Cr-labelled 11RX-infected P815 because spontaneous release of ^{51}Cr was consistently lower with 11RX-infected than C5-infected cells. Figure 5 shows that infection with 11RX induced low levels of antigen-specific Lyt-2$^+$ CTL in the peritoneal cavity and challenge with a second dose of live *Salmonella*, particularly the more persistent C5 organisms, increased the activity of these cells so that approximately 50% of the killing detected using the LDCC assay could be attributed to antigen-specific CTL. Standard methods of

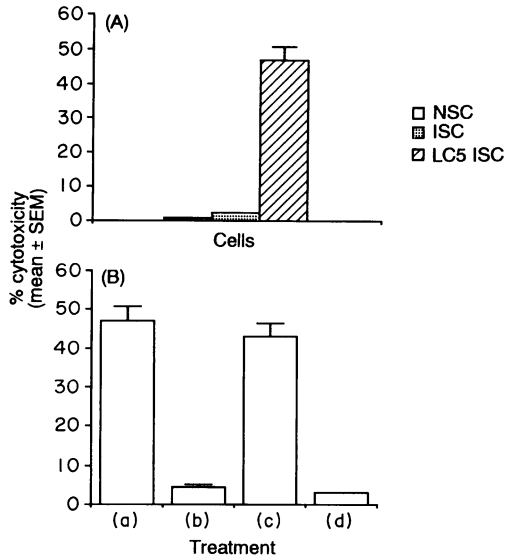

Figure 7. The lytic activity and phenotype of *Salmonella*-specific CTL in cultures of Con A-activated spleen cells. (A) Normal SC (NSC) and SC taken 4 days after 11RX infection (ISC) or secondary challenge with live C5 (LC5 ISC) were activated by 3-day culture with Con A before determining their cytotoxicity using ^{51}Cr-labelled 11RX-infected P815 targets at an effector:target ratio of 25:1. The data obtained are expressed as percentage cytotoxicity (mean ± SEM) of quadruplicate samples. [Cytotoxic activity against Con A-treated ^{51}Cr-labelled P815 cells was 51% (NSC); 55% (ISC) and 76% (LC5 ISC). Maximum cytotoxic activity against untreated ^{51}Cr-labelled P815 cells was 2%.] (B) Spleen cells taken 4 days after secondary challenge with live C5 were cultured for 3 days and then pretreated with (a) complement; (b) anti-Thy-1.2 + complement; (c) anti-L3T4 + complement; or (d) anti-Lyt-2.2 + complement before testing their cytotoxic activity against ^{51}Cr-labelled 11RX-infected P815 at an effector:target ratio of 25:1. The data obtained are expressed as percentage cytotoxicity (mean ± SEM) of quadruplicate samples. (Maximum cytotoxic activity against untreated ^{51}Cr-labelled P815 cells was 3%.)

characterization confirmed that they belonged to the Lyt-2$^+$ T-cell subset (Fig. 6).

The cytotoxic activity of spleen cells of *Salmonella*-immunized mice

LDCC assays indicated that NW-fractionated SC from mice 4 days after primary or secondary infection with *Salmonella* had no cytotoxic activity (data not shown). However, because antigen-specific Lyt-2$^+$ T-effector cells able to mediate DTH were present in SC of mice with a secondary C5 infection,[11] it seemed possible that, although cells with cytotoxic potential were present in the spleen, they did not express cytolytic activity because they were not activated. To investigate this possibility, SC obtained from normal and 11RX-infected mice and mice given a secondary challenge with live C5 were activated by *in vitro* culture with the non-specific T-cell mitogen Con A prior to testing their cytotoxic activity. LDCC assays established that activation of CTL had been achieved since all the cultured cell suspensions exhibited considerable amounts of cytotoxicity mediated by T cells expressing the Lyt-2$^+$ marker, with no evidence for L3T4$^+$ CTL (data not shown).

Incubation of the Con A-activated SC suspensions with ^{51}Cr-labelled 11RX-infected P815 target cells revealed that *Salmonella*-specific Lyt-2$^+$ CTL were induced in the spleens of mice only after secondary infection with C5 organisms. Con A-activated normal SC and SC obtained 4–10 days after injection of 11RX exhibited the same levels of cytotoxic activity as those detected using uninfected ^{51}Cr-labelled P815 (approximately 5% maximum). In contrast, SC harvested 4 days after a secondary challenge with live C5 and cultured *in vitro* with Con A for 3 days to activate them showed more than 40% cytotoxicity (Fig. 7a), accounting for about half of the cytotoxicity detected using the LDCC assay (data not shown). Again, the effectors were Lyt-2$^+$ T cells (Fig. 7b).

DISCUSSION

The results obtained in this study confirm the data presented in the companion paper which indicated that *Salmonella*-specific Lyt-2$^+$ T cells were induced during infection of mice with *Salmonella* and could be detected in PC and SC suspensions from such animals.[11] The finding that *Salmonella*-specific Lyt-2$^+$ CTL were detected in the SC suspensions of mice with a secondary C5 infection only after they were activated *in vitro* with Con A is reminiscent of the report that Lyt-2$^+$ CTL could be detected in spleen cells of *Listeria*-infected mice only after they had been cultured *in vitro* with irradiated, infected accessory cells and IL-2.[12]

The levels of *Salmonella*-specific Lyt-2$^+$ CTL detected in both the PC and SC suspensions never equalled those detected using the LDCC assay, suggesting that a major proportion of the CTL detected was not specific for *Salmonella* antigens. However, it is possible that the system employed in these studies did not detect all the *Salmonella*-specific CTL, because a significant proportion of the P815 cells in the 11RX-infected P815 population did not contain bacteria and, therefore, were probably not expressing *Salmonella* antigens in the context of class I MHC molecules (data not shown). Numerous attempts to modify our current method of infecting target cells have not provided a more uniformly infected target cell suspension and a different approach has to be devised to determine whether all CTL present after immunization with *Salmonella* are antigen-specific.

Because *Listeria* (and *Mycobacteria*) infections also induce CTL that are class II restricted,[14,20] it was somewhat surprising to find that *Salmonella* infections did not induce such a response, despite their ability to induce L3T4$^+$ T effector cells.[8,10] A detailed examination of the role(s) that cytotoxic cells play(s) in the clearance of primary and secondary infections may establish the reason and/or significance of this observation. It has been proposed that the L3T4$^+$ and Lyt-2$^+$ CTL detected in mice infected with *Listeria* and *Mycobacteria* may be responsible for the lysis of infected cells which are unable to kill them, thereby releasing the organisms for uptake and killing by activated macrophages.[20] The need for both types of CTL for this purpose has not been confirmed and the finding that macrophages infected with *M. microti* had reduced levels of expression of class II MHC products,[21] suggests that class I-restricted CTL may be sufficient for the clearance of this infection. A similar situation may apply in *Salmonella* infections. In other words, since all cells express class I MHC molecules, there may be no need for L3T4$^+$ CTL to be induced (but this does not explain *why* they are not induced).

If the CTL do play a role in the immunity to *Salmonella* infection, the appearance of Lyt-2$^+$ CTL in the peritoneal cavities of mice early after primary and secondary infection with *Salmonella* suggests that they may participate in the clearance or control of bacterial growth in the early stages of infection or that they are induced when relatively large numbers of live organisms are present. Even though the kinetics of induction of CTL by other intracellular parasites has not been well characterized, it may be significant that the T cells used to establish *Listeria*-specific cytotoxic T-cell clones were harvested from mice reasonably early (6–8 days) after infection with *Listeria*.[12,14]

Finally, although this is the first report that *Salmonella* antigen-specific CTL are induced during infection with these organisms, it must be emphasized that these results are not unexpected in view of the work referred to earlier[15,16] and the very recent demonstration[22] that recombinant strains of *S. typhimurium* expressing three different antigens (a peptide from *P. berghi*, the nucleoprotein of influenza A virus or ovalbumin) do induce Lyt-2$^+$ CTL[15,22] or L3T4$^+$ CTL.[16] The reasons why one of these induces MHC class II-restricted CTL while the others induce MHC class I-restricted CTL has not been defined, but may well reflect the assay systems used. In other words, as in the present studies, it is possible that CTL of both phenotypes were induced, but they were not detected because of the assays used.

ACKNOWLEDGMENTS

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