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Local Transfer of Delayed-Type Hypersensitivity after *Salmonella* Infection in Mice

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An adoptive local transfer system has been used to study the mediators of delayed-type hypersensitivity induced in mice by infection with *Salmonella enteritidis* 11RX. The cells which transfer this state of hypersensitivity to untreated recipients are nonadherent T lymphocytes with the surface phenotype Lyt 1^+2^-, and successful transfer requires compatibility at the *I-A* subregion of the *H-2* complex. In these and other respects these cells are indistinguishable from those previously found to be responsible for in vitro lymphokine release upon culture with 11RX antigens.

It is now generally accepted that antigen-specific T lymphocytes play a critical role in the development of immunity to intracellular bacterial parasites (5, 16, 23). These cells function by releasing lymphokines which increase the number and bactericidal activity of the tissue macrophages (5, 23). Although this state of macrophage "activation" is relatively transient, the animal remains immune to reinfection by virtue of the longevity of its memory T cells (5, 23).

Studies with various intracellular bacterial parasites have sought to characterize the T cells responsible for resistance and for the state of delayed-type hypersensitivity (DTH) which usually accompanies the development of a protective immune response. Recent work by Kaufmann and Hahn suggests that the T cells generated in mice by *Listeria* infection are pluripotential; antigen-specific clones were developed which proliferated and released lymphokines when cultured with specific antigen in vitro and mediated DTH and protection in vivo (14). These clones comprised Lyt 1^+2^- lymphocytes which were restricted by products of the *I-A* subregion of the major histocompatibility complex (MHC; 13, 14). In contrast, other studies of listerial infection have suggested that the cells responsible for immunity to rechallenge are Lyt 1^-2^+ and *H-2K* restricted in the mouse (3) or correspondingly $OX8^+$ (4) and (primarily) *RT1.A* restricted (12) in the rat. Furthermore, studies with mycobacteria (18, 24) and salmonellae (15, 22) suggest that resistance and DTH are mediated by separable populations of effector cells. Clearly further studies in these areas are required.

To date, the effectors of resistance and DTH to *Salmonella* spp. have not been characterized. Previous work in our laboratory has shown that mice can be protected against challenge with highly virulent *S. typhimurium* C5 by prior infection with avirulent *S. enteritidis* 11RX (6, 7), and we are using this system to study the T cells induced by this infection. In a recent report, we described the capacity of lymphocytes induced by 11RX infection to release lymphokines upon in vitro culture with specific antigen (2); we intend to relate lymphokine release to protective activity in the hope that the former might offer a convenient correlate of the latter. Obviously it is also of interest to investigate the relationship between the expression of DTH and of resistance and to thereby determine whether these are properties of the same cells. Although DTH is readily demonstrable after 11RX infection (6, 28), our attempts to transfer this state of hypersensitivity systemically to naive recipients have been unsuccessful. We have used peritoneal or spleen cells obtained early or late after infection, and attempted to boost the number of DTH effector cells by pretreatment of mice with cyclophosphamide, all to no avail (unpublished data).

To study the generation of DTH effector cells we have therefore used an adoptive transfer system which involves the injection of sensitized cells and specific antigen into the footpads of unimmunized recipients. Others have shown that the swelling response observed in such recipients offers an excellent correlate of the classical DTH response seen in actively immunized mice, in terms of the kinetics, histology, and antigenic specificity of the reaction (20, 25). In this report we use the adoptive local transfer system to characterize the lymphoid cells obtained from mice at various times after 11RX infection.

MATERIALS AND METHODS

Bacteria and bacterial antigen. *S. enteritidis* 11RX was grown to late log-phase in nutrient broth (Difco) and then diluted appropriately for immunization purposes. A cytoplasmic protein extract from 11RX (11RX antigen) was prepared as previously described (28) and used to elicit footpad hypersensitivity reactions.

Mice. (BALB/c × C57BL/6)F_1 (F_1), BALB/c, C57BL/6, and CBA mice were bred in our department and used at 2 to 5 months of age. B10, B10.A(4R), B10.A(5R), and B10.MBR mice were a generous gift from I. McKenzie, Pathology Department, University of Melbourne, Australia. F_1 mice (generally) or C57BL/6 mice (one study) were immunized by intraperitoneal (i.p.) or intravenous (i.v.) administration of ~0.1 50% lethal doses of 11RX (1×10^5 or 2×10^4 organisms, respectively) in 0.2 ml of saline.

Irradiation and cyclosporin A (CyA) treatment. In some experiments mice were subjected to 500 rads or 750 rads of X-irradiation, delivered at a dose rate of ~100 rads per min from a Philips Deep X-ray unit operating at 250 kV.

CyA (Sandoz) was a gift from P. Alexander. The drug was supplied as a solution in oil at 100 mg/ml; this was diluted to 25 mg/ml with olive oil and given orally at a dose rate of 200 mg/kg. Mice received CyA (or olive oil as a control) 24 h before, immediately before, and 24 h after footpad challenge with 11RX antigen.

Lymphoid suspensions. Peritoneal cells (PC) and the non-

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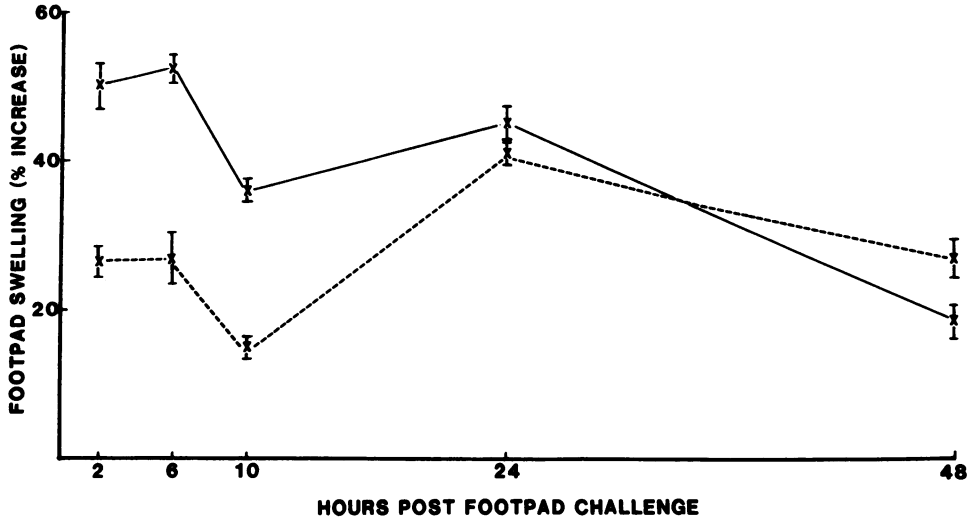

FIG. 1. Kinetics of footpad swelling responses induced by 11RX antigen in actively immunized mice and in unimmunized recipients of sensitized cells. Classical DTH responses were induced in F_1 mice by footpad challenge with 11RX antigen seven days after i.p. infection with 11RX (-----). Other mice from the same group were sacrificed, and 10^7 PC were transferred (with antigen) into the footpads of untreated syngeneic recipients (——). Points on graphs show mean ± SE of six to seven mice per group.

adherent fraction derived therefrom were obtained as previously described (2). Spleen cell (SC) suspensions were prepared as described elsewhere (1), except that erythrocytes were lysed by treatment with ammonium chloride (0.14 M NH_4Cl in 0.017 M Tris buffer [pH 7.2] on ice for 5 min).

Local transfer of lymphoid cells and measurement of footpad swelling. Lymphoid cells were suspended to the desired concentration in Hanks balanced salt solution containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Calbiochem) and mixed with an equal volume of 11RX antigen (at 400 μg/ml in the same medium). Samples (0.05 ml) of this suspension (containing 10 μg of 11RX antigen) were injected through a 27-g hypodermic needle into the right hind footpads of recipient mice, and at various times thereafter, the thicknesses of both hind feet were measured to the nearest 0.05 mm by using dial-gauge calipers (Type 130/3, Mercer, St. Albans, England; the main spring was removed before use to minimize the pressure applied to the foot). Footpad swelling was expressed as percentage increase in the thickness of the injected foot relative to the size of the control foot. Donor and recipient mice were always sex matched and were syngeneic unless otherwise stated. In each experiment, control recipients received either 11RX antigen or sensitized cells alone; the mean swelling observed in such mice was always <10% and is therefore results were not always given below.

DTH in immunized mice was assessed similarly, by determining the footpad swelling induced after injection of 10 μg of 11RX antigen in 0.05 ml of Hanks balanced salt solution containing 10 mM HEPES.

Treatment of lymphoid cells with antibody and complement. The cells responsible for the adoptive local transfer of DTH were characterized by selectively depleting lymphoid cell suspensions using antibody and complement (C'). The antibodies used were (i) an anti-Thy 1.2 serum prepared by immunizing AKR/J mice against CBA thymocytes as described elsewhere (11); (ii) a monoclonal anti-Thy 1.2 antibody (Australian Monoclonal Development, Artarmon, New South Wales, Australia); (iii) a monoclonal anti-Lyt 1.2 antibody (New England Nuclear Corp., Boston, Mass.); and (iv) a monoclonal anti-Lyt 2.2 antibody produced by the HO 2.2 cell line (obtained from K. Lafferty). The properties of these antibodies and the method used for treatment of PC are given elsewhere (2).

RESULTS

Initial studies were designed to validate the use of the adoptive local transfer system as a correlate of the DTH response observed in mice immunized with 11RX. Both reactions involved an infiltrate of mononuclear cells and were inducible with 11RX antigen but not with the control proteins bovine serum albumin or keyhole limpet hemocyanin (data not shown). The kinetics of the swelling observed in the recipients of sensitized cells and 11RX antigen was similar to that elicited in actively immunized mice (Fig. 1). In both situations, two peaks in the footpad swelling response were observed; an early peak at 2 to 6 h preceded the 24- to 48-h peak classically observed in DTH reactions (as reported by others, see reference 27). To avoid any possible contribution of Arthus-type reactions, the delayed peak was used to assess DTH in both systems.

As reported in other systems (29), DTH to 11RX antigens can be prevented by prior X-irradiation, and the results in Table 1 show that the adoptive local transfer of DTH is similarly abrogated by irradiation of recipient mice before cell transfer. The radiation sensitivity of these swelling reactions is due to their dependence upon an influx of marrow-derived monocytes (19, 29); the antigen-specific lymphocytes are not inactivated by this dose of radiation, as cells obtained from immunized mice after irradiation retain an undiminished capacity to transfer DTH locally to untreated recipients (data not shown).

The expression of classical DTH can also be inhibited by administration of the immunosuppressant CyA (26). This drug prevents the initiation of immune responses by inhibiting the production of interleukin 2 (IL2) by T lymphocytes (8). In our systems it prevented the elicitation of DTH by 11RX antigen and the transfer of hypersensitivity to recipient mice (Table 1, experiment 3). On the basis of the above studies, we concluded that the adoptive local transfer system

TABLE 1. Effects of X irradiation and CyA upon DTH response and its adoptive local transfer

Expt[a]	Treatment[b]	Footpad swelling[c]	
		Donors	Recipients
1	None	48.3 ± 2.9	
	X irradiation	2.3 ± 0.6	
2	None		33.2 ± 1.4
	X irradiation		2.8 ± 0.0
3	None	29.4 ± 2.3	40.9 ± 2.1
	CyA	2.2 ± 1.0	9.6 ± 1.3

[a] F_1 donor mice were actively immunized by i.v. infection with 11RX on day 12 (expt. 1) or i.p. infection on day 8 (expt. 3). Recipient mice received 10^6 nonadherent peritoneal cells from donors infected i.p. on day 75 (expt. 2) or i.p. on day 8 (expt. 3).

[b] Mice received 750 rads X-irradiation or CyA as described in Materials and Methods.

[c] Figures show percent increase in footpad thickness measured at 24 h in recipients and at 48 h in donors (mean ± standard error [SE] of results for five or six mice).

provides a valid and convenient means of studying the effectors of the classical DTH response induced by immunization with live 11RX.

Capacities of lymphoid suspensions to transfer DTH. Splenic and PC suspensions were prepared from mice which had been infected i.v. or i.p. with 11RX, and tested for their capacities to transfer DTH locally to untreated recipients. Figure 2 shows the result of one such experiment, in which each recipient received 10^7 (unfractionated) cells. Despite the relative paucity of lymphocytes in PC compared with SC suspensions, the former were consistently more effective, whether the donor mice were immunized i.v. (Fig. 2) or i.p. (data not shown). This difference was not attributable to the NH_4Cl treatment of the SC (see Materials and Methods), as similar treatment of PC did not reduce their activity in this system (data not shown).

PC suspensions harvested 3 or 10 weeks after infection were more active than those obtained after 1 week (Fig. 2). Since other experiments had shown that the capacity to transfer DTH was a property of the nonadherent subpopulation of PC (see below), it was of interest to determine whether changes in the size or activity of this fraction were responsible for the greater activity of PC suspensions collected later after infection. F_1 mice were immunized i.v. or i.p. with 11RX, and nonadherent PC were isolated 1, 3, and 10 weeks later and tested for their capacities to transfer DTH. The cells obtained 3 or 10 weeks after infection were about three times more effective than those collected at 1 week (Fig. 3).

Although the route of immunization (i.v. or i.p.) did not influence cellular activity, the i.p. route was generally used because a higher yield of PC could be obtained; for the same reason, cells were usually harvested 3 to 5 (rather than 10) weeks after infection. In some experiments, however, the

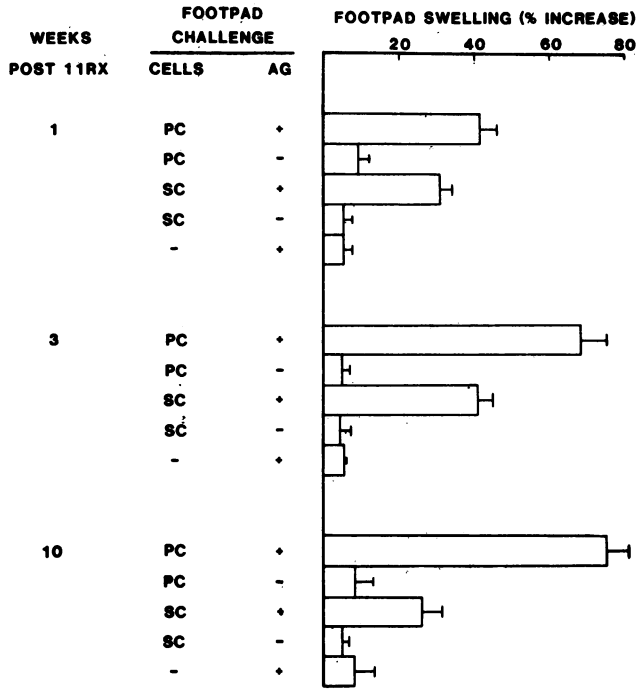

FIG. 2. The capacities of lymphoid suspensions to transfer DTH. F_1 mice were immunized i.v. with 11RX, and at various times thereafter PC and SC were isolated and tested for their capacities to transfer DTH to naive syngeneic recipients in the presence or absence of 11RX antigen (AG). Histograms show the percent increase in footpad thickness (mean ± SE of five mice per group).

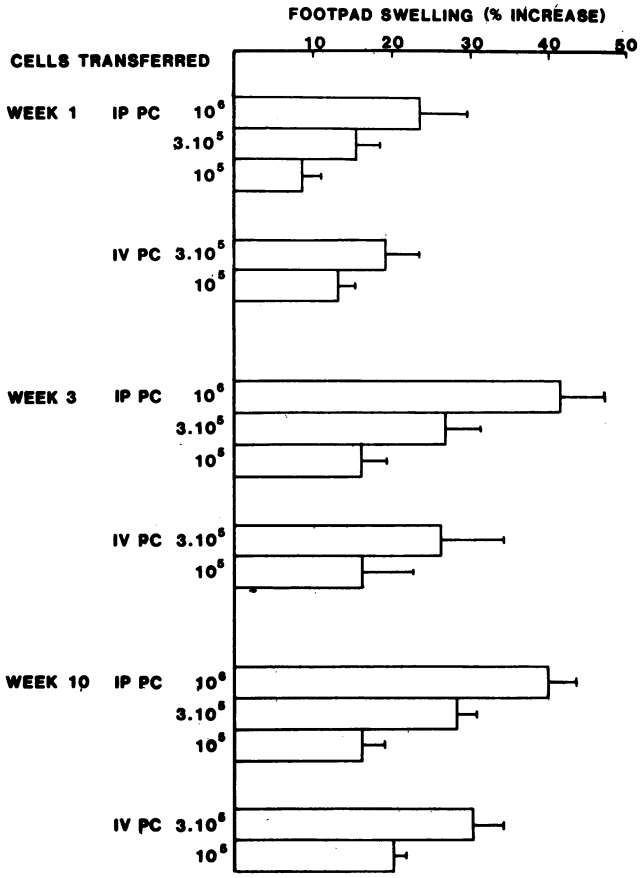

FIG. 3. The local transfer of DTH by nonadherent sensitized cells. F_1 mice were immunized i.v. or i.p. with 11RX; 1, 3, and 10 weeks later, nonadherent peritoneal cells (IV PC or IP PC, respectively) were isolated and transferred in various numbers, together with 11RX antigen, into the footpads of naive syngeneic recipients. Histograms show the percent increase in footpad thickness (mean ± SE of five mice per group). Control mice injected with PC or antigen only displayed minimal footpad swelling (<5%).

i.v. route of infection was employed, but proteose-peptone induction was then used to increase the yield of cells from the peritoneal cavity. (We had previously found that on a cell numbers basis the activity of the induced PC was equivalent to that of the resident population; unpublished data.)

Transfer of DTH is MHC restricted. Subsequent experiments showed that unfractionated PC could transfer DTH to syngeneic or allogeneic mice, but that transfer by the nonadherent PC subpopulation was successful only in syngeneic or semisyngeneic recipients (Table 2). Presumably this reflects the fact that lymphokine release by T cells specific for 11RX antigen is dependent upon an MHC-restricted interaction with antigen-presenting accessory cells (2); in the present context, lymphokine release in the footpad of the recipient would be a prerequisite for mononuclear cell infiltration. It is worth noting that the kinetics of the swelling responses observed in syngeneic recipients were the same irrespective of the presence or absence of nylon-wool adherent cells (data not shown), suggesting that Arthus-type reactivity was not a contributing factor.

It might seem surprising that nonadherent PC isolated from immunized F_1 mice transferred DTH much more effectively to mice of one parental strain than to mice of the other (Table 2, experiment 2). Since C57BL/6 mice can be immunized with 11RX, and display typical footpad swelling responses after receipt of syngeneic sensitized cells (data not shown), this observation indicates an asymmetry in the pattern of *H-2*-restriction of the sensitized F_1 T cells. This finding supports our previous suggestion that during 11RX infection in F_1 mice bacterial antigens might be preferentially presented in the context of $H\text{-}2^d$ MHC products on the surfaces of accessory cells, thus generating a bias in the restriction pattern of the responding T cells (2).

Because of this observation, and the availability of B10 recombinant mouse strains, C57BL/6 (rather than F_1) mice were used to define the *H-2* locus which restricts the adoptive local transfer of DTH. C57BL/6 mice were immunized i.p. with 11RX, and their nonadherent PC were subsequently transferred to recipients of the strains shown in Table 3. Homology of the *K* and *I-A* regions of the *H-2* complex was sufficient for the transfer of hypersensitivity, but homology of *K* alone was not, suggesting that it is the product of the *I-A* subregion which serves as the restricting element. (The higher background response seen in the allogeneic BALB/c mice presumably reflects an incomplete removal of donor accessory cells. This was a consistent problem in two repeat experiments with C57BL/6 mice as sources of sensitized lymphocytes; since there was no such problem with F_1 mice as donors, this might be related to the more protracted 11RX carrier state in C57BL/6 mice.)

TABLE 3. Mapping of the MHC subregion which restricts DTH transfer

Recipient strain[a]	*H-2* haplotype[b]					DTH response[c]
	K	A	J	E	D	
B10	b	b	b	b	b	45.4 ± 4.4
B10.A(4R)	k	k	b	b	b	12.3 ± 1.1
B10.A(5R)	b	b	k	k	d	37.0 ± 2.2
B10.MBR	b	k	k	k	q	12.8 ± 3.3
BALB/c	d	d	d	d	d	14.6 ± 3.5

[a] Recipient mice received 11RX antigen and 10^6 nonadherent sensitized cells isolated from PC harvested from C57BL/6 mice 35 days after i.p. immunization with 11RX.

[b] The regions of homology between donor and recipient mice are underlined.

[c] Figures are percent footpad swelling 24 h after cell transfer (mean ± SE of results for five mice per group).

Attempted systemic transfer of DTH. Having established that nonadherent PC collected late after infection with 11RX are the most active source of effectors of the DTH response, an attempt was made to use such cells to transfer DTH systemically. In addition to untreated syngeneic recipients, cells were injected i.v. into similar mice which had undergone X irradiation just before cell transfer, or which had been treated with cyclophosphamide; both of these procedures have been shown to inactivate precursors of T-suppressor cells in other systems (9, 10, 17). However, only modest swelling responses were elicited by footpad challenge with 11RX antigen (Table 4), compared to the reaction observed after the local transfer of 10-fold fewer cells to untreated recipients.

Surface phenotype of effector cells. The cells responsible for the adoptive local transfer of DTH were characterized by determining their sensitivities to inactivation by various antibodies in the presence of complement. An anti-Thy 1.2 serum eliminated the effector cells, although a monoclonal anti-Thy 1.2 antibody did not (Table 5), even though the latter was more active in terms of complement-dependent lysis of thymocytes (2). Presumably this antibody is directed against an epitope which is not well expressed on sensitized T cells. The cells which transferred DTH were Lyt 1^+2^- (Table 5).

TABLE 2. Transfer of DTH is MHC restricted

Expt	Cells transferred[a]	Recipient strain	Footpad swelling[b]
1	Unfractionated PC	BALB/c × C57BL/6	41.1 ± 1.0
	Unfractionated PC	CBA	39.0 ± 0.7
	Nonadherent PC	BALB/c × C57BL/6	39.9 ± 1.7
	Nonadherent PC	CBA	5.8 ± 0.5
2	Nonadherent PC	BALB/c × C57BL/6	40.9 ± 0.3
	Nonadherent PC	BALB/c	40.9 ± 2.0
	Nonadherent PC	C57BL/6	11.5 ± 1.1
	Nonadherent PC	CBA	4.6 ± 0.9

[a] In both experiments PC were obtained from (BALB/c × C57BL/6)F_1 mice 21 days after i.p. infection with 11RX. Recipient mice received 11RX antigen and either 10^7 unfractionated or 10^6 nonadherent PC.

[b] Figures are percent increase in footpad thickness (mean ± SE of results for five mice per group).

TABLE 4. Attempted systemic transfer of DTH response

Recipient groups[a]	Footpad swelling[b]	
	24 h	48 h
1. Untreated	7.7 ± 1.2	12.7 ± 2.2
2. X-irradiated	4.6 ± 0.6	9.2 ± 2.1
3. Cyclophosphamide treated	14.1 ± 2.3	13.0 ± 1.5
4. Untreated: local transfer	39.1 ± 1.8	11.9 ± 2.0

[a] Peritoneal cells were harvested from F_1 mice 21 days after i.p. infection with 11RX and depleted of excess adherent cells by incubation on plastic dishes. These cells were then transferred systemically (10^7 cells, groups 1 to 3) or locally (10^6 cells, group 4) to syngeneic mice. The recipients were either untreated, X irradiated (500 rads given 2 h before cell transfer) or cyclophosphamide-treated (200 mg/kg, given i.p. 2 days before cell transfer).

[b] Antigenic challenge was given at the time of cell transfer (group 4) or 4 h later (groups 1 to 3). Figures are percent increase in footpad thickness (mean ± SE of results for five mice per group).

TABLE 5. Surface phenotype of the cells which transfer DTH

Pretreatment of PC[a]	Footpad swelling in recipients[b]	
	Expt 1	Expt 2
C′ only	30.8 ± 1.9	33.7 ± 3.2
Monoclonal anti-Thy 1.2 + C′	22.4 ± 1.0	ND[c]
Anti-Thy 1.2 serum + C′	ND	3.9 ± 0.8
Monoclonal anti-Lyt 1.2 + C′	6.6 ± 0.9	8.4 ± 0.7
Monoclonal anti-Lyt 2.2 + C′	28.8 ± 1.8	30.1 ± 1.1

[a] Peritoneal cells (PC) were obtained from F_1 mice infected i.v. with 11RX on day −26 and given 2 ml 10% (wt/vol) proteose-peptone i.p. on day −2 (expt. 1) or from mice infected i.p. on day 35 (expt. 2). Pretreatments were performed as described in the Materials and Methods.

[b] Figures are percent footpad swelling (mean ± SE of results for four or five mice).

[c] ND, Not determined.

DISCUSSION

An adoptive local transfer system has been used to study the mediators of DTH induced by 11RX infection. Preliminary experiments confirmed that this system provides a convenient and consistent correlate of the DTH observed in actively immunized mice. In both cases the footpad swelling responses are characterized by mononuclear cell infiltration and are elicited only by specific antigen; both reactions are inhibited by prior X irradiation or by administration of CyA (Table 1). The kinetics of the two reactions were also similar, with early and late peaks in both swelling responses (Fig. 1).

The cells which transfered DTH to 11RX antigens were sensitized, nonadherent T cells with the surface phenotype Lyt 1^+2^- (Tables 2 and 5). Successful transfer required *H-2* compatibility between donor and recipient, and experiments with B10 recombinant strains of mice suggested that this restriction is imposed by the *I-A* subregion (Tables 2 and 3). Previous studies have shown that 11RX infection generates cells with the propensity to release lymphokines upon subsequent in vitro culture with 11RX antigens and that these cells are also nonadherent Lyt 1^+2^- cells which are *I-A* restricted (2). In the latter situation, the products of the *I-A* subregion govern the interaction between the lymphokine-releasing T cells and adherent, antigen-presenting accessory cells. Given our understanding of the role of lymphokines in the establishment of a DTH response, it seems probable that the same cellular interaction imposes the requirement for *H-2* compatibility in the adoptive transfer of DTH.

There are other similarities between the T cells responsible for IL2 release and those which mediate the local transfer of DTH. In both assays, PC populations are more effective than SC, and the nonadherent fraction of the PC, which includes the effector cells, becomes more active with time after i.p. immunization (Fig. 2 and 3; reference 2). Furthermore, if nonadherent cells from actively immunized F_1 ($H\text{-}2^{b/d}$) mice are tested for their capacities to transfer DTH to mice of either parental strain, or to release lymphokine upon coculture with accessory cells from either strain, then in both systems the effector cells are much more active in the presence of antigen-presenting cells of $H\text{-}2^d$ haplotype (Table 2; reference 2).

It therefore seems probable that the potential to release IL2 in vitro provides a reliable indicator of in vivo DTH activity; indeed the two assays might be detecting the same population of cells, for Kaufmann and Hahn have isolated *I-A*-restricted, Lyt 1^+2^- clones which release IL2 in vitro and transfer DTH locally to naive recipients (13, 14). These clones were much less effective in mediating systemic transfer of DTH (14) and resistance (13), paralleling our own inability to transfer DTH systemically with cell populations which are effective when injected locally (e.g., Table 4).

At least three factors could contribute to the difficulty of transferring DTH systemically. First, there might be suppressor cells in the recipients which modify the effector function of the infused cells (17); despite attempts to inactivate any such suppressor cells we still failed to transfer an appreciable level of DTH systemically (Table 4). Second, it is possible that the infused cells do not circulate properly in the recipient. Although clones which have been maintained in vitro for long periods have been found to display altered migratory patterns in vivo (13), this should not be relevant to the present studies. It has recently been suggested that there is a correlation between Lyt phenotype and the propensity to extravasate into inflammatory foci (21), and as Lyt 1^+2^- lymphocytes were found to be defective in this respect, we are currently examining the significance of this work in our own system. Finally, we have recently obtained evidence that following the infusion of sensitized lymphocytes the nature of the antigen used to elicit footpad responses can have a dramatic effect upon the swelling reaction observed. Cells which are unable to transfer DTH systemically as judged by footpad reactions elicited by soluble 11RX protein antigens, are found to be effective if Formalin-killed 11RX organisms are used as the eliciting stimulus (unpublished data). The underlying basis for this effect is also being investigated.

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