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Immunology

Heterogeneity of the cytotoxic response of thymus-derived lymphocytes after immunization with influenza viruses

(cell-mediated immunity/specificity/H-2 compatibility/thymus-derived lymphocyte memory/secondary response)

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ABSTRACT **Immunization of mice with serologically distinct type A influenza viruses results in development of highly crossreactive populations of cytotoxic thymus-derived lymphocytes (T cells). This can be shown both at the level of effector function and of priming for an enhanced response after challenge with another type A virus. Cytotoxic activity is demonstrable, in both the primary and secondary situations, only for H-2 compatible interactions. Further analysis by competitive inhibition experiments indicates that some of the T cell clones generated are specific for the virus used to immunize, while others are much less restricted. Secondary stimulation may result in preferential stimulation of the crossreactive T cells if the type A viruses used are very different serologically. When more closely related viruses are used, however, some degree of specificity is seen for the challenge virus. Even so, the patterns of crossreactivity observed are complex, and cannot be readily predicted on the basis of known serological relationships between surface hemagglutinin and neuraminidase antigens of type A influenza viruses.**

Cytotoxic thymus-derived lymphocyte (T cell) populations generated after immunization with type A influenza viruses mediate lysis of target cells infected with the same or with serologically distinct type A influenza virus (1). Similar crossreactivity has been described for the oncornavirus (2). Otherwise, T cell effector function and circulating antibody have, to date, shown comparable patterns of specificity. Reciprocal exclusion of cell-mediated cytotoxicity (CMC) is found after infection with influenza type A, influenza type B, vaccinia, ectromelia, and rabies viruses (refs. 1 and 3, and unpublished data). The same is true for paramyxoviruses, poxviruses, and arenaviruses (4, 5). Influenza thus offers novel possibilities for investigating T cell recognition, using a lytic virus that is an important cause of human disease. The present paper is concerned with analyzing the range of T cell specificities that result from both primary and secondary exposure to type A influenza viruses.

MATERIALS AND METHODS

The techniques used have been described (1, 6) and are presented here in brief form. All mice were purchased from the Jackson Laboratories (Bar Harbor, Maine).

Viruses. The virus strains were PR8 [A/PR/8/34 (HON1)], Bel [A/Bellamy/42 (HON1)], AA [A/Ann Arbor/23/57 (H2N2)], HK [HK/X31 (H3N2)] (7), and BLee, a type B influenza virus. Virus stocks were stored frozen as allantoic fluid containing between 1200 and 3000 hemagglutinating (HA) units/ml (8). Mice were injected intraperitoneally with a single dose of 120–300 HA units. The target cells were exposed to 6–15 HA units of type A influenza virus per 10^6 cells, or to 0.2 HA units of BLee per 10^6 cells.

Cytotoxic Assay. Target cells were labeled with ^{51}Cr, infected with influenza virus for 1 hr at 37°, and dispensed into plastic trays at a concentration of 1.5×10^4 cells per 6-mm diameter well in 100 μl of medium (RPMI 1640 containing 10% fetal calf serum). Immune spleen cells, depleted of erythrocytes, were added in a further 100 μl of medium and samples were incubated for 12 hr at 37°. Unless otherwise stated, the spleen populations were obtained from CBA/J mice and the targets were L929 fibroblasts (L cells), both of which express H-2^k antigenic specificities. Results are given as per cent mean specific ^{51}Cr release for four replicates (6).

Competitive Inhibition of Cytotoxicity. The specificity of the virus-immune T cell response was assessed by competing for cytotoxic effector function by interposing different ratios of unlabeled, virus-infected cells (9, 10). The competitors were exposed to virus 4 hr before the ^{51}Cr-labeled cells, and were thus expressing virus-induced cell-surface changes earlier. Other experiments (unpublished) indicate that maximum antigen expression does not occur for 7 hr after infection. The ^{51}Cr-labeled targets were dispensed first, overlaid with competitors in 100 μl of medium, and the spleen cells were then added in a further 100 μl. This was all done at room temperature. The time between each step ranged from 30 to 90 min, so cells had ample time to settle to the bottom of the well before the next cell population was added. The samples were then incubated for 12 hr at 37°, and 100 μl of the supernate was removed for γ counting. The ^{51}Cr-labeled targets had thus been held for a total of 13 hr at 37° after infection, the unlabeled competitors for 17 hr.

RESULTS

Crossreactivity of effector T cells

The development of primary and secondary cytotoxic responses was compared for mice of the same age and sex (Fig. 1). CMC resulting from primary immunization of CBA/J mice with HK virus peaks on day 5 and is crossreactive for L cells infected with type A, but not with type B, influenza viruses. This specificity pattern is also apparent when mice primed with PR8 (HON1) are later challenged with HK (H3N2). However, the overall magnitude of lysis is considerably greater after secondary stimulation, and capacity to mediate maximal ^{51}Cr release is generated 2 days earlier than in the primary response. Furthermore, in both situations, CMC is recognized only when target cells share H-2 (mouse major histocompatibility) antigenic specificities with the mouse strains in which the lymphocytes are sensitized (Tables 1 and 2). Lysis may thus be considered to reflect activity of T cells because this constraint is not known to apply to other categories of CMC (4). Also,

Abbreviations: CMC, cell-mediated cytotoxicity; H, influenza virus hemagglutinin antigen; N, influenza virus neuraminidase antigen; HA unit, hemagglutinating unit; H-2, mouse major histocompatibility complex; T cell, thymus-derived lymphocyte.

Table 1. Cytotoxic T cells generated after primary immunization with HK virus interact only with H-2 compatible virus-infected target cells

Mouse strain	H-2 type K	H-2 type D	% ^{51}Cr release from virus-infected targets: L cells (H-2^k) PR8	L cells (H-2^k) HK	P815 (H-2^d) PR8	P815 (H-2^d) HK
B10.Br	k	k	52	97	0	0
B10.A	k	d	41	71	37	20
B10.D2	d	d	4	8	35	32

Mice were immunized intraperitoneally 5 days previously and the data given are for a ratio of 100 spleen cells:1 target cell.

Table 2. Maximal cytotoxic activity generated on secondary challenge is restricted to H-2 compatible interactions

Mouse strain	H-2 type K	H-2 type D	% ^{51}Cr release from virus-infected targets: L cells (H-2^k) PR8	L cells (H-2^k) AA	L cells (H-2^k) HK	MC57G (H-2^b) PR8	MC57G (H-2^b) AA	MC57G (H-2^b) HK
CBA/J	k	k	68	51	100	0	13	6
B10.A	k	d	49	47	90	0	0	0
B6	b	b	0	0	0	68	20	95

Mice were primed with AA (H2N2) and challenged after 28 days with HKX31 (H3N2). Spleen cells (100:1) were assayed 5 days later on PR8 (HON1), HKX31, or AA virus-infected target cells.

previous experiments (1, 3) have shown that all cytotoxicity is removed by complement-mediated lysis of both primarily and secondarily stimulated influenza-immune lymphocyte populations treated with antibody specific for T cells.

Immunization with influenza virus results in generation of crossreactive T cell populations. This can be shown both at the level of cytotoxic effector function and of priming for a secondary response.

Specificity of primary T cells

A more detailed analysis of the specificity of primary cytotoxic T cells was made by using unlabeled virus-infected cells to selectively compete for effector function (Fig. 2). Some general patterns emerge: (*a*) Specificity is, as would be expected, always greatest for the homologous situation. This is particularly obvious for the interaction between the AA-immune spleen and the AA target, where the AA competitor is by far the most effective. (*b*) Inhibition of cytotoxicity tends to be maximal for the unlabeled competitor that is infected with the same virus as the ^{51}Cr-labeled target cell. For example (Fig. 2), the results for the Bel targets and PR8 or HK spleens indicate that immunization with these viruses generates minority T cell populations that interact best with cells infected with Bel virus. (*c*) The next most effective competitor is, for heterologous interactions, the cell infected with the virus used to immunize. (*d*) The spectrum of crossreactivity is not obviously related to serologically defined hemagglutinin (H) or neuraminidase (N) antigens of influenza virus.

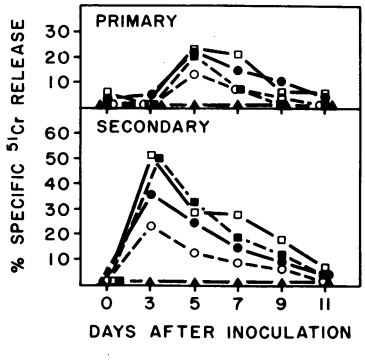

FIG. 1. Time course of the primary and secondary CMC responses: ♂ CBA/J mice were immunized with PR8 and challenged 52 days later (secondary) with HK. Previously unexposed mice of the same age (16 weeks) were also inoculated with HK (primary). Spleen cells were assayed (100:1) on L cells infected with PR8 (HON1) (□), Bel (HON1) (■), AA (H2N2) (○), HK (H3N2) (●), and BLee (▲) influenza viruses.

Do competitors that cause comparable levels of inhibition [e.g., cells infected with Bel and AA in the PR8 spleen, PR8 target interaction (Fig. 2)] express common or different crossreacting antigens? Various populations of unlabeled competitors were mixed, but there was no indication of additive effects (Table 3). It thus seems that at least some of the crossreactive T cell populations may be recognizing a shared antigen, present

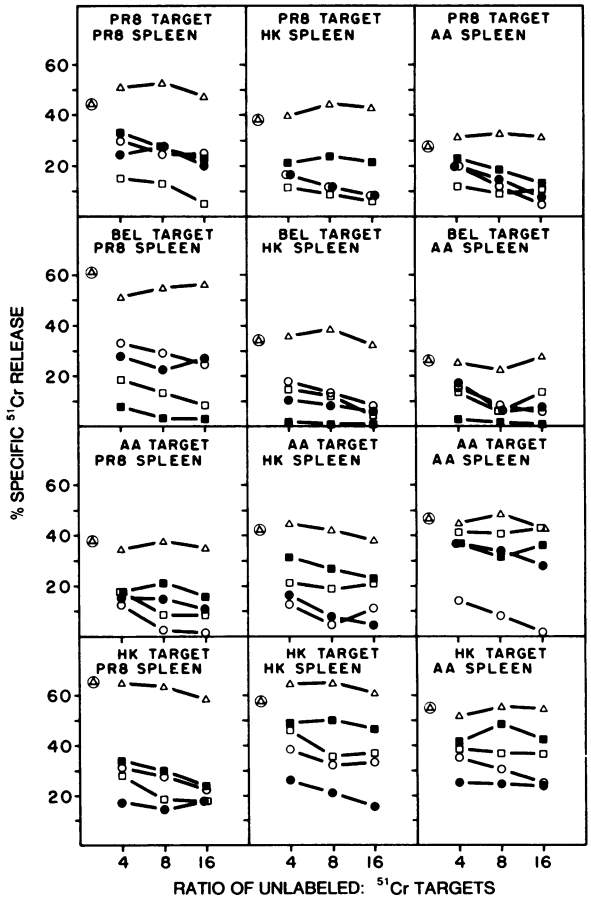

FIG. 2. Competitive inhibition of cytotoxicity in the primary response: CBA/J spleen cells (100:1) were assayed at 5 days after immunization with PR8, AA, or HK. The unlabeled L cells were infected with PR8 (HON1) (□), Bel (HON1) (■), AA (H2N2) (○), and HK (H3N2) (●) influenza viruses. Effector function was also measured in the presence of normal L cells (△) and in the absence of competitors (◬). All assays were done on the same day with the same cell populations.

Table 3. Effect of mixing different populations of competitor cells on cytotoxicity mediated by HK-immune lymphocytes

Competitor populations	% ^{51}Cr release for HK targets	
	4:1*	8:1
Normal	69	69
HK (H3N2)	39	28
PR8 (HON1)	59	47
PR8 + AA	58	51
AA (H2N2)	52	51
AA + Bel	49	53
Bel (HON1)	62	62

* Ratio of unlabeled competitor cells to ^{51}Cr-labeled L cells. The mixtures (PR8 + AA and AA + Bel) included equal numbers of each cell type. Spleen cells (CBA/J) were overlaid at 100:1.

on the surface of cells infected with many type A influenza viruses.

Secondary stimulation of crossreactive T cells

Challenge of PR8 (HON1)-primed mice with HK (H3N2) apparently selects for the crossreactive T cell population(s) (Fig. 3). Both at 3 and 5 days after secondary stimulation, competitors infected with PR8 or HK cause almost equivalent inhibition of cytotoxicity for the HK target. The shared antigen(s) recognized is evidently not the chicken host component, which is common to egg-grown type A and B influenza viruses (11). Cells infected with BLee do not compete (Fig. 3), and no indication of a secondary response is seen when mice primed with BLee are challenged with HK or PR8 (Table 4). Furthermore, restimulation with the same influenza virus does not give rise to CMC (PR8 → PR8, Table 4). Prior exposure induces development of circulating antibody (6), which presumably neutralizes the challenge virus. The crossreactive T cells cannot, therefore, be recognizing some unknown contaminant (whether virus or mycoplasma) present only in the embryonated eggs used to prepare all the type A (but not the BLee) virus stocks.

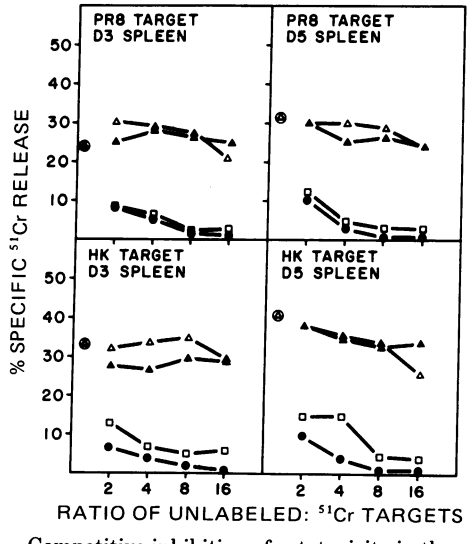

FIG. 3. Competitive inhibition of cytotoxicity in the secondary response: CBA/J mice were primed with PR8 and challenged after 24 days with HK. Spleen cells (50:1) were assayed (◎) 3 or 5 days later in the absence of competitors on L cells infected with PR8 or HK. The unlabeled competitors were normal (△) or infected with PR8 (HON1) (□), HK (H3N2) (●), or BLee (▲) influenza viruses.

Table 4. Secondary cytotoxic response is not directed against the chicken host component shared by types A and B influenza viruses

Primary	Virus challenge	Day	% ^{51}Cr release	
			PR8	HK
Nil	PR8	5	43	39
Nil	PR8	3	7	8
BLee	PR8	3	9	5
HK	PR8	3	69	68
PR8	PR8	3	2	2
Nil	AA	5	52	72
Nil	AA	3	6	9
BLee	AA	3	8	8
HK	AA	3	97	117*
PR8	AA	3	90	106*

B10.Br (H-2^k) mice were challenged with PR8 (HON1) or AA (H2N2) at 26 days after primary immunization, and spleen cells were assayed (50:1) 3 or 5 days later on L cells infected with PR8 or HK (H3N2).

*% ^{51}Cr release by immune lymphocytes was greater than water lysis.

Specificity patterns in the secondary response

Restimulation of PR8 (HON1)-primed mice with HK (H3N2) or of AA (H2N2)-primed mice with PR8 (HON1) induces highly crossreactive secondary T cell responses (Figs. 3 and 4). However, challenge of AA-primed mice with HK results in CMC which, at both 3 and 5 days after secondary stimulation, is more specific for HK than for AA or PR8 (Fig. 4). The same spectrum of crossreactivity was observed when these experiments were repeated, using *in vivo* (unpublished) and *in vitro* (Table 5) techniques for restimulation. This difference between specificity patterns after challenge of AA-primed mice with PR8 or HK cannot be attributed to the shared neuraminidase antigen (N2) of HK and AA because competitors infected with AA are no more effective (Fig. 4 and Table 5) than those infected with PR8 (N1).

By serological criteria, however, H2(AA) and H3 (HK) are more similar to each other (13, 14) than to HO (PR8). Perhaps there is some correlation between influenza H antigen type and T cell recognition (14). Our results (Fig. 4 and Table 5) indicate that exposure to H2 may generate memory T cells more reactive for H3 than for HO. Even so, any conclusion in this regard is premature. Such relationships need to be investigated further for a range of type A influenza viruses and for genetic recombinants that express different combinations of H and N antigens (15).

DISCUSSION

The essential point emerging from these experiments is that, within the type A influenza viruses, specificity patterns in the cytotoxic T cell response cannot be readily predicted on the basis of known serological relationships between viral hemagglutinin and neuraminidase antigens. Perhaps the T cell repertoire differs from that for antibody (16). However, evidence exists from other systems that binding sites on T cells and on free immunoglobulin molecules express identical idiotypes, and may thus be coded for by the same V genes (genes coding for immunoglobulin variable region) (17, 18). It is possible that the repeated arrangement of multiple recognition units in a stable matrix (the lymphocyte plasma membrane) may give rise to a spectrum of reactivity that is quite different from that defined

Table 5. Specificity of lymphocytes from AA-primed mice after restimulation *in vitro* with PR8 or HK influenza virus

Challenge virus	Competitor cells	% ^{51}Cr release from virus-infected L cells					
		PR8 (HON1)		AA (H2N2)		HK (H3N2)	
		8:1*	16:1	8:1	16:1	8:1	16:1
PR8	Normal	31	26	28	25	70	56
PR8	PR8	16	16	8	3	34	28
PR8	AA	15	14	4	2	40	32
PR8	HK	16	15	3	1	34	21
HK	Normal	36	33	35	34	69	73
HK	PR8	17	16	12	9	49	46
HK	AA	21	19	5	2	45	40
HK	HK	17	17	1	0	33	26

Spleen cells from B10.A mice primed with AA 56 days previously were restimulated *in vitro* (3), using a stimulator:responder ratio of 1:1, and assayed (50:1) 6 days later.

* Ratio of unlabeled competitors to ^{51}Cr-labeled target cells.

by serum immunoglobulin (19). An alternative explanation is that the crossreactive T cells recognize a type-specific determinant. The type A influenza viruses share internal ribonucleoprotein and matrix components, the latter comprising 40% of the virus protein (15, 20). Neither antigen has been detected on the surface of the virion or on the outside of the cell plasma membrane. This may, however, be analogous to the situation for oncornaviruses. There are indications that oncornavirus-immune T cells recognize p30, a group-specific internal virus protein (2, 21). This was considered somewhat of an enigma, but it is now known that a glycoprotein with the antigenicity of p30 is expressed on the surface of virally modified cells (22).

It seems that, in influenza, we are considering a range of T cell specificities, some restricted to a particular virus and others highly crossreactive for a range of viruses. Further subdivision of the cell-mediated immune response into distinct clonotypes is, however, not yet feasible. We cannot rigorously distinguish whether slight differences in crossreactivity patterns are due to operation of a variety of T cell subsets or reflect variation in the extent to which individual type A viruses induce expression of a common cell surface modification. Resolution of this question requires a better understanding of the antigens recognized. The problem is currently being approached by immunizing with various components of influenza virus (15, 20).

Even at the present stage of analysis, however, it is apparent that exposure to a single virus results in proliferation of many T cell clones. Cytotoxic lymphocytes are, for the type A influenza viruses, readily subdivided into a minimum of two categories—the one specific and the other crossreactive. Previous studies with the lymphocytic choriomeningitis model established that different populations of T cells are associated with the K and D regions of the H-2 gene complex (4, 10). Furthermore, experiments with H-2 mutant mice indicate that more than one virus-immune T cell clone is associated with each of these H-2 loci (23). Manifestations of cell-mediated immunity in any given virus infection (4) apparently reflect the net consequence of a very heterogeneous T cell response.

The present findings may also resolve two apparent paradoxes raised by earlier experiments. The first concerns the difficulty of blocking cytotoxic T cells with antibody specific for the virus (4, 16). It is now obvious that the spectrum of recognition differs for the two systems, the cell-mediated effect being much more crossreactive than antibody. The second stems from evidence that the T cell response to trinitrophenyl-modified lymphocytes is subject to some form of immune response

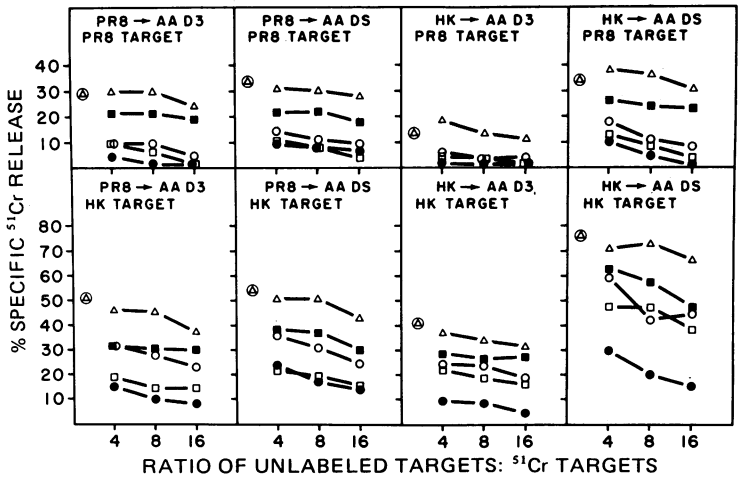

FIG. 4. Specificity of the secondary cytotoxic response at 3 or 5 days after inoculation of CBA/J memory mice with PR8 or HK; primary immunization was with AA, given 28 days before challenge. Spleen cells were assayed (100:1) in the absence of unlabeled competitors (Ⓐ) or in the presence of unlabeled normal L cells (△) or L cells infected with PR8 (HON1) (□), Bel (HON1) (■), AA (H2N2) (○), or HK (H3N2) (●) influenza viruses.

(Ir)-gene control, though no comparable role for Ir genes is demonstrable in the virus systems (24, 25). Any Ir gene effect specific for a particular viral antigen would, however, tend to be masked by concurrent T cell responses associated with the infectious process.

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