GENERATION OF BOTH CROSS-REACTIVE AND VIRUS-SPECIFIC T-CELL POPULATIONS AFTER IMMUNIZATION WITH SEROLOGICALLY DISTINCT INFLUENZA A VIRUSES*

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Immune thymus-derived lymphocytes (T cells) generated during the course of virus infections of mice have, in most instances, shown predictable specificity patterns (1). Reciprocal exclusion of cytotoxic T-cell activity has been demonstrated for three diverse groups of viruses: arenaviruses [lymphocytic choriomeningitis virus (LCMV)[1]], poxviruses (ectromelia and vaccinia), and paramyxoviruses (Sendai). The only aberrant finding is that cytotoxic T cells from mice infected with LCMV or vaccinia virus lyse trinitrophenyl-modified lymphocytes (2).

Virus-immune T cells also exhibit another order of specificity, which could not have readily been foreseen. Effector T-cell function, as measured by both in vitro and in vivo criteria, is recognized only when virus-infected targets share *H-2K* or *H-2D* genes with the mouse strain in which the lymphocytes are sensitized (3–6). Such is the case for both the normal physiological situation, and for radiation chimeras in which *H-2*-different T cells apparently see alloantigen as self (7–10). Either virus-immune T cells recognize both self (*H-2*) and nonself (virus), or the lymphocyte receptor is specific for some neoantigen determined by both host and viral genomes (11, 12).

Various speculations have been advanced to explain this *H-2* restriction phenomenon (7, 11, 12) At one extreme is the idea that the T cell expresses two variable (V) genes, one specific for self-*H-2* and the other for virus (12). The most drastic alternative is the possibility that the infectious process results in derepression of cryptic host genes, and reactivity is directed solely against abnormally expressed alloantigens (13). This latter concept implies that the T cell does not recognize virus at all. It thus becomes important to compare T-cell responses resulting from exposure to different viruses of similar molecular biology.

The influenza viruses offer an ideal experimental system for this purpose. Firstly, there is the possibility of using A and B strain influenza viruses, which bear a common host determinant (if grown in chick embryo) but are otherwise serologically distinct (14). Fine specificity can then be studied within the A

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[1] *Abbreviations used in this paper:* B cell, antibody-forming cell precursor; CMI, cell-mediated immunity; H, influenza virus hemagglutinin; HA, hemagglutinating; LCMV, lymphocytic choriomeningitis virus; M, influenza virus matrix; RIA, radioimmunoassay; RNP, influenza virus ribonucleoprotein; V, variable.

strain viruses, which express different hemagglutinin (H) and neuraminidase surface antigens but share internal ribonucleoprotein (RNP) and matrix (M) components (15). We have thus investigated T-cell responses to a variety of influenza A viruses, representing subtypes first isolated from man in 1933 (H0N1), 1957 (H2N2), and 1968 (H3N2).

Materials and Methods

Mice. CBA/J, C57BL6/J (B6), C57BL/10 (B10), B10.A, B10.A(5R), B10.BR, and CBA/J × B6 F_1 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. B10.A(2R) and B10.A(4R) mice were bred in colonies maintained at the Wistar Institute by Dr. D Gotze.

Viruses The influenza A virus strains PR8 [A/PR/8/34 (H0N1)], Bel [A/Bellamy/42 (H0N1)], AA [A/Ann Arbor/23/57 (H2N2)], and NT60 [A/Northern Territory/60/68 (H2N2)] and the influenza B strain (BLee) were originally obtained from Dr. S. Fazekas de St. Groth, Division of Animal Genetics, CSIRO, Sydney, Australia. The virus strains Hick [A/Hickox/40 (H0N1)] and HK/X31, a recombinant between PR8 and a Hong Kong strain which shows the antigenic characteristics of the Hong Kong virus (16) were supplied by the Center for Disease Control, Atlanta, Ga. and Dr R. G. Webster, St Jude Childrens' Research Hospital, Memphis, Tenn., respectively. Virus stocks of high infectivity titer were grown in the allantoic cavity of embryonated chicken eggs, and stored frozen at −70°C. All such stocks contained between 1,200 and 3,000 hemagglutinating (HA) U/ml (17).

Immunization. Mice were generally immunized intraperitoneally (i.p) with 1.0 ml of a 1.10 dilution (in phosphate-buffered saline) of allantoic fluid containing virus (100–300 HA U per mouse) In one experiment mice were anesthetized with chloroform, and given 50 μl of a 1:10,000 dilution intranasally

Cytotoxic T-Cell Assay. The methods used are similar to those described for other viruses (18). Briefly, L929 fibroblasts (L cells) were grown in RPMI 1640 (Flow Laboratories, Inc , Rockville, Md.) containing 10% fetal calf serum and antibiotics. This medium was used throughout. Cells were trypsinized, pelleted, washed, and labeled for 1.5–2 h at 37°C with $Na^{51}Cr$ (New England Nuclear, Boston, Mass.) at a concentration of 250 μCi/10^7 cells. The cells were then pelleted again and resuspended in medium containing virus for 1 h at 37°C, washed twice in medium, and dispensed into 96-hole plates (Linbro Chemical Co., New Haven, Conn.) in 100 μl of medium to give 1.5×10^4 cells per well The concentration used for the A strain viruses was 5.0 ml of a 1·10 dilution of stock allantoic fluid in medium per 2×10^7 cells (approximately 50 HA U per 10^6 cells), while a 1:300 dilution (0.5 HA U per 10^6 cells) was used for the BLee.

The target cells were then overlaid with the lymphocyte populations, in a further 100 μl of medium, and the assays were held overnight at 37°C in a humidified atmosphere containing 7% CO_2 in air Assay supernates (100 μl) were then removed for γ-counting. The initial assays were incubated for 16 h, but background levels of ^{51}Cr release were found to be rather high (from 30 to 40%) All experiments are now done using a 12 h assay which gives a background, for normal spleen cells or medium, ranging from 20 to 30% of hydrolysis. The water lysis value is determined by adding 100 μl (1.5×10^4 cells) of the target cell population to 1.9 ml of distilled water, incubating this with the assay, and then measuring the number of counts present in 1.0 ml of the supernate

All results are expressed as mean percent specific ^{51}Cr release for replicates of three or four wells. Standard errors within the groups were reproducibly less than 5%, and generally below 2%, and are not shown for clarity of presentation of results The formula (1) used for calculating percent specific ^{51}Cr release is (It-Nt) × 100/Wt-Nt, where W is water lysis, t is the target, I is immune lymphocytes, and N is normal lymphocytes. Uninfected L cells were carried as controls in many experiments, but were not killed by immune spleen cells.

Lymphocytes Immune spleen and lymph node cell populations were processed and depleted of erythrocytes as described previously (1) Viability was determined by trypan blue exclusion, and all ratios quoted in the results are adjusted to viable cell counts. Some lymphocyte preparations were depleted of B cells by passage through nylon wool columns (19), and T cells were removed by incubation with a rabbit anti-mouse brain serum [anti-T, (20)] and guinea pig complement This serum, which has been used extensively by other workers (8), was kindly supplied by Dr. D. Gotze.

Radioimmunoassay. Unlabeled, virus-infected L cells were prepared and plated into wells as described for the cytotoxic assay. After overnight incubation they were fixed with 0.15% glutaraldehyde and were used as immunoadsorbents in a radioimmunoassay (RIA) as described by Segal and Klinman (21). This involved incubation of the immunoadsorbent with an appropriate dilution of mouse serum, followed by quantitation of the bound mouse Ig by means of ^{125}I-labeled rabbit-anti-mouse antibody

Results

Specificity Between Type A and B Influenza Viruses and Vaccinia. Mice were immunized i.p. with large doses of influenza virus and spleen cells were assayed for effector function on virus-infected L cells. Maximal cytotoxic activity was observed at 5 days after exposure to PR8 (H0N1) or BLee, and the response was specific for the immunizing virus (Table I). Reciprocal exclusion of lytic function was also observed for PR8 and vaccinia virus (Table II). The specificity demonstrated is thus of the same order as found previously for other viruses (1).

Cross-Reactivity Between Type A Viruses. Reciprocal priming with different strains of influenza A viruses revealed a pattern of complete cross-reactivity (Table III). The different viruses varied in their immunogenic capacity: effector lymphocytes from mice given PR8, HKX31, and NT60 were the most active. However, all populations were lytic for the H0N1-, H2N2-, and H3N2-infected target cells. No clear indication was found of preference for the homologous interaction.

This result was somewhat surprising as Cambridge et al. (23) had found previously that cytotoxic lymph node cells from mice infected with influenza A viruses show specificity for the virus H antigen. Differences from the present study are that the effectors were not identified as T cells and that only one strain (A/WSN, H0N1) was used for immunization and tested on targets infected with a variety of viruses. Another possible source of discrepancy is that virus given i.p. in large quantities may be processed in an unphysiological way, with resultant generation of aberrant T-cell specificities. Cytotoxic assays were thus made using mediastinal lymph node cells from mice infected intranasally with much lower doses of virus. Again the same specificity pattern was observed, with complete cross-reactivity between the type A viruses, but reciprocal exclusion of cytotoxicity for BLee (Table IV).

All viruses used in the present study were grown in the allantoic cavity of the chick embryo. Virus particles produced in this way are known to express a chicken host component (14), which is common to influenza A and B strain viruses and normal allantoic fluid. Both the reciprocal exclusion of cytotoxicity for influenza A and B viruses and the fact that mice immunized with allantoic fluid did not generate effector capacity for either influenza virus-infected targets or for L cells previously incubated with the normal allantoic fluid (Table V) indicates that the cross-reactivity observed for A strain viruses is not due to immunization with a common antigen of chicken origin.

Also, serum antibodies detected in mice immunized by the procedure used to generate cytotoxic spleen cells did not show any significant cross-reactivity. Significant binding of antibody was recognized only for target cells infected with the virus used for immunization (Table VI). Apparently the virus-infected L cells used in this assay do not express any cell surface antigen common to PR8

TABLE I
Specificity of Cell-Mediated Lysis for Influenza A and B Viruses

Immune* spleen	Days after inoculation	% ^{51}Cr release from virus-infected L cells			
		PR8		BLee	
		50:1	100·1	50 1	100:1
PR8	3	3	15	4	0
	5	48	64	4	9
	7	25	28	5	5
BLee	3	0	0	25	36
	5	0	2	32	49
	7	11	7	20	18

* B10.BR mice were inoculated i.p. with a 1:10 dilution of allantoic fluid containing influenza virus Assays were incubated for 16 h at 37°C

TABLE II
Reciprocal Exclusion of Cytototoxicity for Influenza and Vaccinia Viruses

Immune spleen*	% ^{51}Cr release from L cells			
	PR8		Vaccinia	
	25:1	50·1	25.1	50.1
PR8	32	40	0	3
Vaccinia	0	0	79	96

* CBA/J mice were immunized i.p. 5 days previously with 250 HA units of PR8 influenza virus, or intravenously with 10^6 $TCID_{50}$ of vaccinia virus (22). The vaccinia-immune population was enriched for T cells by passage through nylon wool (19) The assays were incubated for 12 h at 37°C

TABLE III
Extensive Cross-Reactivity Between Spleen Cell Populations from Mice Immunized with Different A Strain Viruses

Immune spleen*	% ^{51}Cr release from virus-infected L cells				
	PR8 H0N1	Bel H0N1	AA H2N2	NT60 H2N2	HK H3N2
PR8	70	78	31	48	60
Bel	19	16	22	12	28
Hick	38	18	25	16	32
AA	50	33	36	38	51
NT60	58	42	36	46	62
HK	62	41	41	46	50

* CBA/J.mice were immunized i.p. 5 days previously. The results given are for a ratio of 50 spleen cells:1 target cell Data for Hick (H0N1) targets is not shown, as background levels of ^{51}Cr release were >70%.

TABLE IV
Cytotoxic Activity of Mediastinal Lymph Node Cells from Mice with Influenza Pneumonia

Mediastinal* lymph node	% Specific ^{51}Cr release					
	PR8 (H0N1)		HKX3 (H3N2)		BLee	
	25:1	50:1	25:1	50:1	25:1	50:1
PR8	30	51	26	42	0	0
HKX31	20	31	22	31	0	0
BLee	5	3	0	0	41	74

* Mice were dosed intranasally 7 days previously with 50 μl of a 10^{-4} dilution of stock virus. Pneumonia was most severe in those given PR8, and least marked in mice dosed with HKX31.

TABLE V
Cross-Reactivity Does Not Reflect Immunization with Egg Antigens

Immune population*	% ^{51}Cr release from L cells			
	Allantoic fluid	PR8 H0N1	HKX31 H3N2	BLee
Allantoic fluid	0	0	1	0
PR8	0	54	72	4
HKX31	0	50	71	6
BLee	0	0	8	27

* CBA/J × B6 F_1 mice were injected i p. with influenza virus or a comparable (1:10) dilution of normal allantoic fluid Assays (100:1) were incubated for 12 h at 37°C.

TABLE VI
Lack of Serological Cross-Reactivity between Influenza A and B Viruses

Immunizing* virus	Day of sampling	μg of antibody per ml of serum binding to:‡		
		L-PR8	L-HK	L-BLee
PR8 (H0N1)	14	235	7	3
HK (H3N2)	13	8	65	4
PR8 (H0N1)	23	300	4	4
HK (H3N2)	23	9	240	4
BLee	23	5	5	205
Normal serum		4	2	4

* CBA/J mice were immunized by the procedure used to generate cytotoxic T cells.
‡ Virus-infected L-cell monolayers were prepared by the technique used for the cytotoxic T-cell assay, incubated for 16 h at 37°C, and fixed with 0.15% glutaraldehyde for RIA (21).

(H0N1) and HKX31 (H3N2) viruses which is readily demonstrable by serological techniques.

Identity of the Cytotoxic Population. What is the nature of the effector cell in influenza-immune spleen? Cytotoxic activity was considerably enhanced by passing lymphocytes through nylon wool columns (Table VII), which tend to remove antibody-forming cell precursors (B cells) and enrich for T cells (19). The same cross-reactivity pattern was observed for the purified populations. Effector function was totally abrogated by treatment with an anti-T serum (20) and

TABLE VII
Effect of Nylon Wool Depletion and Treatment with Anti-T Serum and Complement

Spleen* population	PR8 (H0N1)		AA (H2N2)	
	12 h	16 h	12 h	16 h
PR8 immune	39	49	13	36
Nylon wool effluent	74	98	34	52
Nylon wool adherent	35	38	14	33
Anti-T + C	0	0	0	3
Complement alone	49	60	9	49
Washed‡	45	68	16	46
AA immune	30	48	11	32
Nylon wool effluent	60	81	39	63
Nylon wood adherent	17	35	10	39
Anti-T + C	0	0	0	0
Complement alone	33	46	22	45
Washed‡	41	60	22	48

* CBA/J × B6 F_1 mice were dosed i.p. 5 days previously. Immune populations were assayed at 100:1, after passage through nylon wool columns (19) or treatment with anti-T serum and complement (20). Approximately 30% of spleen cells were recovered after either treatment

‡ Processed in parallel with the two preceding groups.

TABLE VIII
*Immune Lysis is Maximal When Target and T Cell Share H-2 Genes**

Mouse strain	H-2 type I K ABC SD	% ^{51}Cr release from HK-infected L cells ($H\text{-}2^k$)		
		25:1	50:1	100:1
B10	bbbbbb	6	4	5
B10 A	kkkddd	16	18	18
B10.A(2R)	kkkddb	16	19	20
B10.A(4R)	kkbbbb	13	17	21
B10 A(5R)	bbbddd	0	1	1
B10.Br	kkkkkk	21	28	38
B6	bbbbbb	0	0	0
CBA/J	kkkkkk	22	23	33
CBA/J × B6 F_1		21	27	42

* Mice were immunized i.p. with HKX31 virus and killed 5 days later

guinea pig complement. Also, specific lysis was mediated only by virus-immune spleen cells which share $H\text{-}2K^k$ or $H\text{-}2D^k$ genes with the virus-infected L cells (Table VIII), a constraint that is unique for T cells (7). It thus seems apparent that the effectors operating in these assays are cytotoxic T cells. This confirms earlier findings of Yap and Ada (24) for the A/WSN strain of influenza virus.

Evidence for Different T-Cell Subsets. Subdivision of cytotoxic T-cell specificities with respect to requirement for *H-2K* or *H-2D* compatibility is readily achieved by utilizing "cold-target" competitive-inhibition protocols (25). The same is true for differentiating between the effects of priming with different viruses (Fig. 1). Cross-reactive cytotoxic T-cell activity recognized for the heterologous interaction (e.g., H0N1 → H3N2) is abrogated to the same extent when

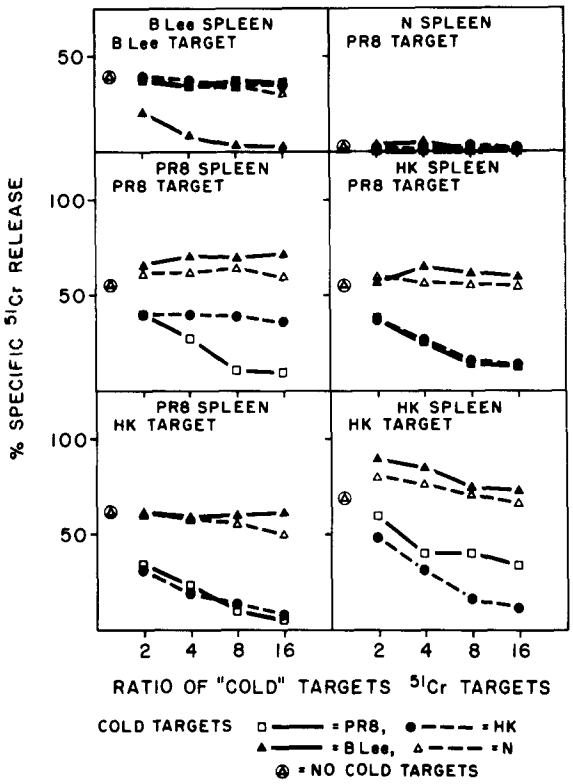

FIG. 1 Inhibition of immune spleen cell effectors (100:1) in a 12 h assay using different ratios of cold, unlabeled competitor cells. The competitors were normal L cells (N), or L cells infected with BLee, PR8 (H0N1), or HKX31 (H3N2) 4 h before the ^{51}Cr-labeled targets were infected with one of these strains of influenza virus

either H0N1 or H3N2 virus-infected, unlabeled cells are interposed between lymphocyte and target. Little inhibition is recognized when normal L cells, or L cells infected with BLee are used as competitors. In the homologous situation (e.g., H0N1 → H0N1), however, much greater inhibition is recognized with the H0N1 competitor than with the H3N2-infected cells. The converse is also true. Apparently at least two populations of immune T cells are functioning, the one being cross-reactive between different A strain viruses, the other specific for the homologous virus. This is the first time that we have been able to subdivide virus-immune T-cell specificities, other than on the basis of requirement for *H-2* compatibility.

Cross-Priming. Further evidence for cross-reactivity between PR8 (H0N1) and HKX31 (H3N2) influenza virus-immune T cells was found when mice primed with PR8 were challenged 3 wk later with HKX31. A second exposure to PR8 resulted in cytotoxic activity less than that observed for primary immunization (Fig. 2). This reduction probably reflects neutralization of the input virus by antibody. Memory PR8 mice challenged with HKX31, however, generate immune spleen cells which are more lytic for both the H0N1 and H3N2 virus-infected target cells. Is cross-priming of T cells central to the "original antigenic

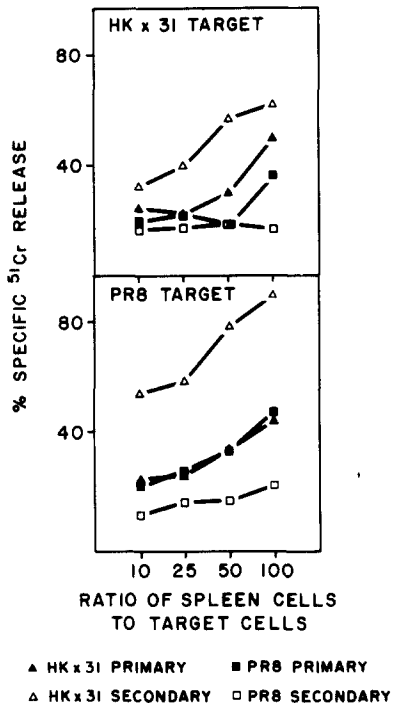

FIG. 2. CBA/J mice were injected i.p. with a 1:10 dilution of allantoic fluid containing PR8 (H0N1) influenza virus. These mice were then challenged (secondary) i.p. 21 days later with the same dose of PR8 or HKX31 (H3N2) influenza virus, and spleen cells were assayed after a further 5 days. Previously unexposed mice were immunized at the same time (primary). The assays were incubated for 12 h at 37°C. Specific ^{51}Cr release caused by memory spleen cells from unchallenged PR8 memory mice was <5%, as were cytotoxic activities of all spleen populations for normal L cells.

sin" phenomenon? Current experiments are concerned with analyzing this question.

Discussion

Recognition that there is extensive cross-reactivity in the cytotoxic T-cell response to different influenza type A viruses raises important questions concerning the role of cell-mediated immunity (CMI) in influenza. Studies with ectromelia and LCMV indicate that cytotoxic, or surveillance (26), T cells are of prime importance in elimination of virus-infected cells in vivo (4–7). Capacity to adoptively transfer effector function in these two diseases correlates closely with cytotoxic activity measured in vivo, and is subject to the same requirement for *H-2K* or *H-2D* identity between stimulator environment and virus-infected target cell, or recipient mouse.

Is this also true for influenza? If so, the fact that widespread exposure of human populations to one A strain influenza virus apparently does not protect against a new, serologically distinct pandemic strain (27) might be thought to mean that CMI plays no significant role in this disease. There is, however, some experimental evidence that mice previously infected with an H0N1 virus sup-

port decreased virus replication on subsequent challenge with an H2N2 strain (28). Other studies indicate that T cells may, as in LCMV (7), mediate immunopathological process (29, 30). Perhaps human influenza reflects both protective and immunopathological consequences of T-cell effector function. May this have been a factor in the extremely high mortality observed in young adults during the 1918 pandemic? Availability of an in vitro correlate for CMI should considerably facilitate an experimental approach to such questions (7, 18).

What are the cross-reactive T cells recognizing? One possibility is that the T-cell receptor is specific for an "altered self" determinant, perhaps an abnormally expressed alloantigen (13), which is common to cells infected with very similar viruses. An alternative is that shared virus components, such as the internal RNP and M protein, may be expressed in some way on the surface of the virus-infected cell. This is, however, thought not to occur (15). Even so, the M protein aligns on the cytoplasmic face of the plasma membrane (15). Could this induce some specific complementary modification, or rearrangement of molecules, on the outside of the lipid-protein bilayer? Such a change would not be detected by antisera directed against M protein purified from egg-grown virus (15).

Another consideration is that this is a rather acute immune response, being maximal at 5 days after primary immunization. Perhaps the specificity of the T-cell receptor is equivalent to that of an early IgM, which may be much less restricted than the late IgG used to serologically define influenza strains (14). The same mechanism [anti-idiotype response? (31)] that regulates IgM production may also prevent further clonal expansion of effector T cells.

The fact that virus-specific T-cell populations can also be demonstrated indicates that at least part of the T-cell repertoire is directed against the virus. Perhaps we are considering a continuum of recognition. We know that a single mouse produces more than one B-cell clone specific for a given H antigen (32). The same V gene products may also be expressed on T cells (33, 34). The binding characteristics, and thus the specificity, of a secreted Ig molecule may be quite different from that of multiple recognition structures [single Ig heavy chains? (33, 34)] arranged in a stable matrix, such as the cell membrane (12). Some T-cell clones may thus be highly cross-reactive, even though free Ig is not, the degree of specificity depending (as always) on the uniqueness of the antigenic site recognized.

The central question is whether we can account for this T-cell specificity pattern in terms of known components of influenza virus. This may be possible. A range of recombinant viruses are available (14), monoclonal antisera can be generated (32), and the various virus proteins can be obtained in pure form (15). Is there any need to invoke an "altered self" concept, other than at the level of associative recognition of virus and H-2 antigen?

Summary

Specificity of cytotoxic T-cell function was investigated for a range of different influenza viruses. T cells from mice immunized with A or B strain influenza viruses, or with vaccinia virus, showed reciprocal exclusion of cytotoxicity. Extensive cross-reactivity was, however, found for lymphocyte populations from mice infected with a variety of serologically distinct influenza A viruses, though

serum antibodies did not cross-react when tested in a radioimmunoassay using comparable target cells as immunoadsorbents. This apparent lack of T-cell specificity was recognized for immune spleen cells generated after intraperitoneal inoculation of high titers of virus, and for mediastinal lymph node populations from mice with pneumonia due to infection with much less virus. The phenomenon could not be explained on the basis of exposure to the chicken host component, which is common to A and B strain viruses. However, not all of the virus-immune T-cell clones are cross-reactive. Competitive-inhibition experiments indicate that a considerable proportion of the lymphocyte response is restricted to the immunizing virus. Even so, the less specific component is significant. Also, exposure to one type A virus was found to prime for an enhanced cell-mediated immunity response after challenge with a second, serologically different A strain virus.

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