Brief Definitive Report

SPECIFICITY OF THE HELPER T CELL FOR THE CYTOLYTIC T LYMPHOCYTE RESPONSE TO INFLUENZA VIRUSES*

By CAROL S. REISS‡ AND STEVEN J. BURAKOFF§

From the Division of Pediatric Oncology, Sidney Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

A role for helper cells in regulating the cytolytic T lymphocyte (CTL) response to virally infected cells was suggested by Zinkernagel and co-workers (1). Recently, Ashman and Mullbacher (2) presented direct evidence that helper cells could augment the CTL response to influenza virus; however, the specificity of these helper T cells was not determined.

The specificity of the Ly-1^+ cells that regulate the delayed-type hypersensitivity (DTH) response has been assessed and appears to be hemagglutinin (HA)-specific (3–5), whereas the CTL appear to demonstrate both HA specificity and subtype cross-reactivity (6–9); these cross-reactive CTL appear to recognize an antigen that is common to the viral type. In this study, the specificity of the helper T cells that regulate the CTL response to influenza was examined.

Materials and Methods

Mice. Female BALB/c ($H\text{-}2^d$) (Charles River Breeding Laboratories, Wilmington, Mass.) mice between the ages of 6 and 10 wk were used.

Viruses. Influenza A/PR/8/34 (HON1), A/Japan/305/57 (H2N2), A/HK/8/68 (H3N2), and B/Lee/40 viruses were originally obtained from Dr. J. L. Schulman (Mt. Sinai School of Medicine, New York) and were propagated in the allantoic cavity of embryonated chicken eggs (Spafas Inc., Norwich, Conn.) for 40 h. The virus preparations were stored as allantoic fluids at −80°C.

Serological Studies. Monoclonal antibodies, purchased from New England Nuclear, Boston, Mass., included anti-Lyt-1.2, Lyt-2.2, and Thy-1.2. Lymphocyte populations were treated twice with the indicated monoclonal antibody (at 10^{-3}, 10^{-4}, and 10^{-4} dilutions, respectively) plus complement. Low-Tox rabbit complement (Cedarlane Laboratories/Accurate Scientific Co., Hicksville, N. Y.) was used at a 10^{-1} dilution in L-15 medium (Microbiological Associates, Walkersville, Md.).

Generation of Primary Anti-Influenza CTL. Helper cells were obtained from mice injected intraperitoneally with cyclophosphamide (100 mg/kg, Cytoxan; Mead Johnson & Co., Evansville, Ind.) 2 d before subcutaneous injection of virus (1,000 HAU) and 6–8 d before killing. Spleen cells of the primed mice were teased with forceps and filtered through nylon mesh to yield a single cell suspension in Eagle's minimal essential medium with Earle's salts (MEM) supplemented with 3% fetal bovine serum (Microbiological Associates). These helper cells were irradiated (900 rad, Gammacell 40; Atomic Energy of Canada). Stimulator lymphocytes were

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obtained from naive syngeneic mice, treated with Tris (15 mM), ammonium chloride (0.83%) solution to lyse erythrocytes, 1,500 rad irradiated, and infected with at least five egg-infectious doses of virus per cell for a minimum of 1 h at 37°C, and then washed three times to remove unadsorbed virus. Responder lymphocytes were from cyclophosphamide-pretreated (100 mg/kg) syngeneic mice. In 16-mm Linbro cultures plates (Flow Laboratories, Inc., Rockville, Md.), varying numbers of the above cells and 900-rad-treated naive spleen cells, used as filler cells, were co-cultured for 4 or 5 d at 37°C in 2 ml of RPMI 1640 (Microbiological Associates), supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), penicillin (100 μg/ml), streptomycin (100 mg/ml) (Gibco Laboratories, Grand Island Biological Co.) 50 μg/ml Gentamicin sulfate (Schering Corp., Kenilworth, N. J.), 10 mM Hepes (Microbiological Associates), and 5×10^{-5} M 2-mercaptoethanol (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.).

Assay of Cytolytic T Cell Activity. Target cells used were uninfected or infected (10–100 egg-infectious doses per cell) P815 ($H\text{-}2^d$) cells. Cells were infected for a minimum of 5 h, pulsed in the last h with 100 μCi $Na_2{}^{51}CrO_4$ (NEZ-030, New England Nuclear), and washed three times before being used as targets. Varying numbers of effector cells cultured as above and 10^4 target cells were combined in MEM supplemented with 10% fetal bovine serum, penicillin/streptomycin solution (Grand Island Biological Co.), and 1% nonessential amino acids (Grand Island Biological Co.) in duplicate 10- × 75-mm glass tubes for 4 h at 37°C. Thereafter, 1 ml of phosphate-buffered saline (PBS; pH 7.0) was added to each tube, samples were centrifuged at 200 *g*, and supernates were decanted for counting in a gamma counter. Spontaneous release was determined by release of label in the presence of normal spleen cells. Total releasable counts were determined by four freeze-thaw cycles of the target cells before the addition of PBS. Percent specific release was determined by the formula: $100 \times \frac{(E - C)}{(F - C)}$, where E is isotope release in tubes containing immune effectors and targets, C is the isotope release in tubes containing normal spleen cells and targets, and F is the maximum isotope release obtained after freeze-thaw of targets. All measurements were performed in duplicate. The standard error of the mean was always <2–3%. Differences of >5% were almost invariably significant at $P < 0.05$.

Results and Discussion

In Vivo Priming Results in the Generation of Radioresistant Helper Cells. To assess whether helper cells could be isolated from in vivo primed mice, spleen cells from mice primed subcutaneously with A/PR/8/34 (PR8) virus 7 d previously were irradiated and added to culture with naive spleen cells (responder cells). Varying numbers of primed or naive irradiated cells were added to yield a constant cell number of 1×10^6 per culture well with 1×10^6 naive responder cells and 3×10^6 irradiated, PR8 virus-infected stimulator cells. 5 d later, virus-specific CTL activity was determined. Fig. 1 illustrates that the addition of increasing numbers of primed irradiated spleen cells resulted in a proportional increase in virus-specific CTL. In the absence of primed spleen cells, naive cells were unable to differentiate to antiviral CTL. No cytolytic activity was observed if the irradiated "helper" cells were cultured with stimulator cells but without naive responder cells, suggesting that these irradiated helper cells were not directly cytolytic.

Helper Cell Activity Is Type Specific. The specificity of these in vivo generated helper cells was assessed. Spleens from BALB/c mice, immunized with either PR8 or B/Lee virus, were a source of irradiated helper cells and were mixed with naive responder lymphocytes and irradiated PR8 or B/Lee virus-infected syngeneic stimulator cells. The effector cells generated were assayed on P815 targets infected with either virus. Helper cells primed to PR8 virus helped naive cells to respond to PR8 virus but not to B/Lee virus. Conversely, B/Lee virus-primed helper cells enabled naive lympho-

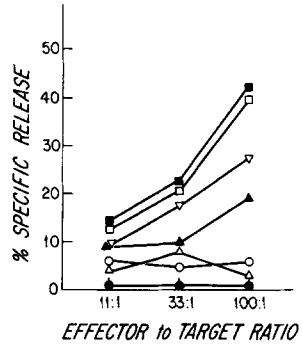

FIG. 1. In vitro generation of virus-specific helper T cell function. BALB/c mice were cyclophosphamide pretreated (100 mg/kg) 2 d before priming; 6–8 d after priming, their spleen cells were irradiated. They were co-cultivated with naive responder BALB/c cells, naive irradiated filler BALB/c cells, and irradiated infected stimulator cells for culture. Assay was performed on infected and uninfected P815 cells. Spontaneous release was 12%. Responder cells, no stimulators (●); responder cells, stimulators (△); responder cells, stimulators plus 1×10^6 naive filler cells (○); responder cells, stimulators plus 7×10^5 naive filler cells and 3×10^5 primed helper cells (▲); responder cells, stimulators, 5×10^5 naive filler cells plus 5×10^5 primed helper cells (▽); responder cells, stimulators, 3×10^5 naive fillers, plus 7×10^5 helper cells (■); responder cells, stimulators, plus 1×10^6 helper cells (□).

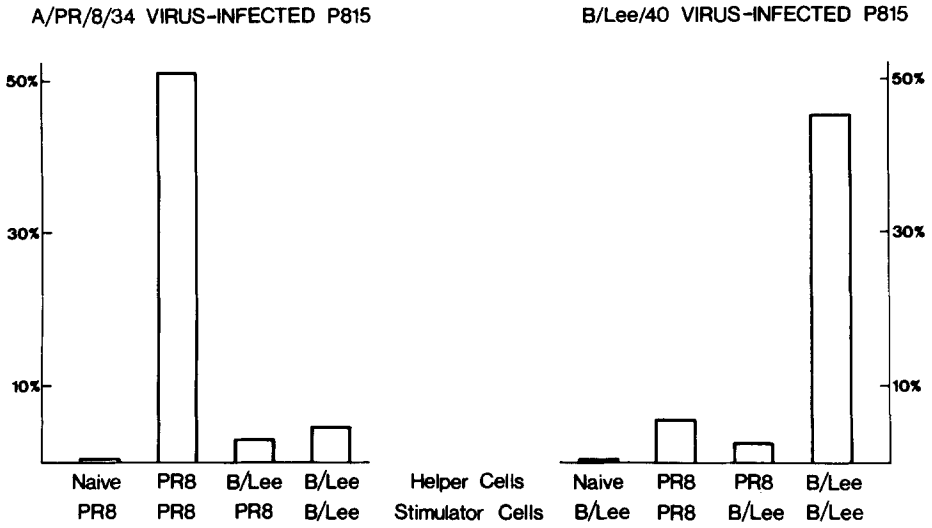

FIG. 2. Type-specific restriction of antiviral helper cells. Helper cells were donated by naive, influenza A/PR/8/34 virus or influenza B/Lee/40 primed BALB/c mice, irradiated, and mixed with naive responder lymphocytes and stimulator cells infected with either PR8 or B/Lee viruses. 5 d later, the effector lymphocytes were assayed on ^{51}Cr-labeled P815 targets. Data shown is at an effector to target ratio of 100:1. Spontaneous release in the 4-h assay was 20% (PR8 infected) and 12% (B/Lee infected). Data are expressed as percent specific release.

cytes to respond to B/Lee virus but not to PR8 virus-infected cells (Fig. 2). Thus, the specificity of helper cells is determined at the level of priming, and is specific for the virus type used.

Both in vivo and in vitro stimulation with influenza virus result in the generation of HA-specific CTL and CTL that are type specific, i.e., they are cross-reactive among the subtypes of type A influenza virus (6–9). The exact nature of the determinant(s)

recognized by these cross-reactive CTL is still controversial (10–17). To determine whether helper cells were HA or type specific, BALB/c mice were primed either with PR8, Japan, or HK virus, which are representatives of different subtypes of influenza A viruses. The irradiated helper cells were subsequently co-cultured with naive responder cells and irradiated stimulator cells infected with either PR8, Japan, or HK virus. In a series of six experiments, a representative of which is shown in Table I, PR8 virus-primed helper cells consistently helped naive lymphocytes to respond to stimulator cells infected with viruses of different subtypes of type A influenza virus. In addition, the specificity of the resultant effector cells was observed to be cross-reactive, as both infected targets could be lysed. Therefore, both at the level of the helper cell and the responder cell, T cells with cross-reactive specificities were demonstrated. Although these would appear to be the predominant CTL, it is possible that a population of HA-specific helpers and CTL exist within this population.

Phenotype of the Helper Cell and CTL. The helper cells were further characterized for theta and Lyt antigens. Table II demonstrates the effect of treatment of helper cells with monoclonal anti-Thy-1.2, anti-Ly-1.2, and anti-Ly-2.2 and complement before their addition to culture. We observed complete elimination of antiviral helper activity with anti-Thy-1.2 or with anti-Ly-1.2 antibody and complement treatment. However, treatment of the helper cells with monoclonal anti-Ly-2.2 antibody and complement resulted in a loss of only 30–50% of the helper function. Thus, whereas some helper T cells are Ly-1^+2^-, it appears that a significant portion of the helper cells express the Ly-1^+2^+ phenotype. The inability to reconstitute a full antiviral response when equal numbers of Ly-1-depleted and Ly-2-depleted primed irradiated helper cells were added to cultures is consistent with this interpretation. It is possible that some helper cells were frozen at the junction between a Ly-1^+2^+ splenic precursor and a Ly-1^+2^- "mature" helper cell at the moment of harvesting and irradiation.

To phenotype the CTL, BALB/c splenic lymphocytes were treated with monoclonal anti-Ly-1.2 or anti-Ly-2.2 and complement either immediately before initiation of

TABLE I

Characterization of the Specificity of the Helper and Effector Cells: Type A Influenza Virus Cross-Reactivity

Responder	Virus-infected stimulator cell	Virus-primed helper cell	Target cells	
			A/PR/8/34 virus-infected P815 cells	A/Japan/305/57 virus-infected P815 cells
Naive	A/PR/8/34	Naive	3	4
Naive	A/PR/8/34	A/PR/8/34	33	47
Naive	A/PR/8/34	A/Japan/305/57	39	38
Naive	A/Japan/305/57	Naive	2	5
Naive	A/Japan/305/57	A/PR/8/34	43	59
Naive	A/Japan/305/57	A/Japan/305/57	38	51
A/PR/8/34 primed	A/PR/8/34	—	56	76
A/PR/8/34 primed	A/Japan/305/57	—	45	78
A/Japan/305/57 primed	A/PR/8/34	—	72	83
A/Japan/305/57 primed	A/Japan/305/57	—	74	82

BALB/c mice were primed with either A/PR/8/34 or A/Japan/305/57 virus 7 d before initiation of culture. Primed cells were irradiated and then combined with irradiated BALB/c stimulator cells that had been infected with either virus, and also naive BALB/c splenic lymphocytes for a 5-d culture. Cytolytic activity was assayed on three ^{51}Cr-labeled P815 target cells: uninfected, influenza A/PR/8/34 virus-infected, or influenza A/Japan/305/57 virus-infected. Data shown is an effector to target ratio of 50:1 with spontaneous releases of 15, 17, and 18%, respectively.

TABLE II

Characteristics of the Helper T Cells

	Treatment of primed helper cells	Number added per well	Specific release
			%
(A)	Complement only	10×10^5	40
	Anti-Thy-1.2 + complement	10×10^5	0
	Anti-Ly-2.2 + complement	10×10^5	23
	Naive helper cells	10×10^5	0
(B)	Complement only	10×10^5	43
	Anti-Ly-1.2 + complement	10×10^5	5
	Anti-Ly-2.2 + complement	10×10^5	18
	Anti-Ly-1.2 + complement	5×10^5	12
	Anti-Ly-2.2 + complement	5×10^5	
	Naive helper cells	10×10^5	0

Monoclonal antibodies and low toxicity rabbit complement were used for two treatment cycles before irradiation and co-cultivation of PR8 virus-primed BALB/c lymphocytes with naive responder cells and PR8 virus-infected stimulator cells. After 5 d culture, effector cells were assayed. Data shown represents lysis of PR8-infected P815 cells at an effector to target ratio of 100:1. Spontaneous release was 24% (A) and 21% (B).

culture with primed irradiated helper T cells or after 5 d of co-cultivation. At both times, all cytolytic activity was eliminated by either monoclonal antibody and complement, demonstrating that the cytolytic effector cell elicited from the naive population expresses the Ly-1^+2^+ surface phenotype. In contrast, primed spleen cells undergoing a secondary in vitro response were found to be almost entirely Ly-1^-2^+ because all CTL activity was susceptible to monoclonal anti-Ly-2.2 and complement treatment and very little CTL activity was lost when cultured cells were treated with monoclonal anti-Ly-1.2 and complement (data not shown).

In conclusion, we have developed a model system in which the generation of CTL requires helper T cells that recognize type-specific determinants on influenza virus. The availability of viral subtypes has allowed us to carry out this genetic analysis. It has recently been demonstrated in the response to an allogeneic H-2 antigen (18) that the antigenic requirements for the triggering of helper T cells differs markedly from the requirements in triggering CTL. The helper cell appears to see processed antigen in the context of the Ia antigen of an antigen-presenting cell. The CTL can, however, see unprocessed antigen without it being presented by an Ia positive antigen-presenting cell. We are now in a position to undertake a similar analysis in the immune response to another well-defined set of antigens: the influenza viruses.

Summary

We have described a model system in which helper T cells are required to mount a primary antiviral cytolytic T lymphocyte response. The radioresistant helper cell can be found in the spleens of mice that have been immunized subcutaneously with influenza viruses 6–8 d previously. These helper cells appear to be type specific but cross-reactive among the subtypes of influenza A viruses. The phenotypes of the interacting cell populations were determined.

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