

EFFECT OF HELPER T CELLS ON THE PRIMARY
IN VITRO PRODUCTION
OF DELAYED-TYPE HYPERSENSITIVITY TO INFLUENZA VIRUS

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A requirement for helper T cells in the production of IgG antibody by B cells has been known for a long time and has been extensively studied (1-3). More recently, evidence has accumulated that there may be two classes of helper T cells for antibody production (4-6) and that T cell-T cell collaboration may be involved in the in vitro generation of cytotoxic T lymphocytes (CTL)¹ against alloantigens (7-10). Moreover, Pilarski and her colleagues (11, 12) have shown that there is an absolute requirement for antigen-specific helper T cells in the generation of CTL from thymocyte precursors, as well as for CTL responses to metabolically inactivated stimulator cells (13). The possible participation of helper T cells in anti-viral CTL responses is suggested from recent work both in vivo (14) and in vitro (15-17). A similar requirement for helper cells in the generation of H-Y-specific CTL has also been suggested by von Boehmer and Haas (18). On the other hand, Finberg et al. (19) have shown that radioresistant helper T cells generated in vivo can augment trinitrophenyl (TNP)-specific CTL activity generated in vitro, and Fujiwara et al. (20) have found a similar situation with respect to cell-mediated immunity (CMI) against syngeneic tumors in vitro. Another major arm of the CMI response is the delayed-type hypersensitivity (DTH) response, and there are now three reports that such activity can be generated in vitro from normal spleen cells against heterologous erythrocytes (21, 22), or to a soluble protein and a synthetic antigen (23). A recent paper has suggested that there is a requirement for T-T cooperation for the manifestation of a DTH response to the synthetic polypeptide poly(LTyr,LGlu)-poly(DLAla)--poly(LLys) [(T,G)-A--L] (24). Earlier work from this laboratory has studied the DTH response to influenza virus as an antigen, both in vivo (25-27) and in vitro (28), and on the possible role of the DTH effector cells in the pathogenesis of viral infection (27). We have recently demonstrated that a weak primary DTH response to influenza virus can be obtained in vitro². In this report, we show that this response can be greatly augmented by radioresistant helper T cells and describe the antigenic specificity, as well as H-2 requirement of the helper cells.

¹ *Abbreviations used in this paper:* CMI, cell-mediated immunity; CTL, cytotoxic T lymphocytes; DTH, delayed-type hypersensitivity; EID₅₀, median egg-infectious dose; HAU, hemagglutinin units; MHC, major histocompatibility complex; Th, helper T lymphocytes; (T,G)-A--L, poly(LTyr,LGlu)-poly(DLAla)--poly(LLys); TNP, trinitrophenyl.

² Leung, K. N., N. K. Mak, and G. L. Ada. 1981. The inductive requirements for the primary in vitro generation of delayed-type hypersensitivity to influenza virus in mice. *Immunology*. In press.

Materials and Methods

Mice. The various strains of inbred mice used in this work were bred at the John Curtin School of Medical Research, Canberra, Australia. For each experiment, mice of the same sex and age (5–9 wk) were used.

Viruses. Influenza virus strains A/WSN (H0N1), A/JAP (H2N2), A/R.I. (H2N2), A/P.C. (H3N2), and B/LEE virus and Sendai virus were grown in the allantoic cavity of 10-d-old embryonated eggs for 40–48 h. Virus titers are usually expressed as hemagglutinating units (HAU). Infectivity of virus was determined by titration in eggs and is expressed as median egg-infectious dose (EID₅₀). Procedures for virus purification and inactivation by exposure to ultraviolet light (uv) irradiation were outlined in detail in earlier reports (25, 26). In some experiments, freshly harvested, concentrated preparations of infectious virus were used for elicitation of DTH responses and methods for preparing these viruses were essentially the same as described elsewhere (29).

Generation of Helper T Cells In Vivo. Mice were injected intravenously with 10 HAU infectious or uv-irradiated virus and, except in kinetic studies, spleens were removed 2 d later and single cell suspensions were made (30) that were γ -irradiated (2,000 rad from a ⁶⁰Co source, 660 rad/min) before use.

In Vitro Generation of Primary DTH Response and Assay of Helper T Cell Activity. 1.5×10^7 normal spleen cells exposed (1 h, 37°C) to either infectious virus (3 EID₅₀/cell) or uv-irradiated virus (1.5×10^3 HAU/ 10^7 cells) were cocultured with 7.5×10^7 normal spleen cells (responder cells) in 40 ml culture medium (Eagle's minimum essential medium [F-15; Grand Island Biological Co., N. Y.], supplemented with 10% fetal calf serum [Commonwealth Serum Laboratories, Melbourne], 10^{-4} M 2-mercaptoethanol and antibiotics [100 U/ml penicillin G, 100 μ g/ml streptomycin, and 100 μ g/ml neomycin]). The cultures were incubated at 37°C in a gas phase of 10% CO₂ in air. Cells were harvested after culturing for 5 d, dead cells were removed by centrifugation over Ficoll-Isopaque gradient (31), and viable cells were tested for DTH activity by adoptive transfer with antigens into naive recipients.

For the assay of helper activity, in vivo primed immune spleen cells (helper cells) were γ -irradiated (2,000 rad), and were then added to the primary cultures at a fixed 5:5:1 ratio of helper:responder:stimulator. The helper activity of the primed cells was assessed as the percent enhancement of the primary DTH response, which was calculated as follows:

$$\frac{\text{Mean increase in footpad thickness in the presence of helper cells in the culture} - \text{Mean increase in footpad thickness in the absence of helper cells in the culture}}{\text{Mean increase in footpad thickness in the absence of helper cells}} \times 100.$$

Adoptive Transfer of DTH and Measurement of Footpad Swelling. Usually, $5\text{--}10 \times 10^6$ primary effector cells generated in vitro were injected with purified, uv-irradiated virus (6×10^3 HAU) into the right hind footpad of the recipient mice in a total volume of 50 μ l. The same amounts of phosphate-buffered saline, pH 7.2, were injected into the left hind footpads as a control. If infectious virus was used for elicitation of the DTH response, the effector cells were always injected 6 h after injection of virus (2.5×10^3 HAU) into the same mice footpads (28). Footpad swelling was measured 24 h later as described previously (26).

Cell Fractionation and Characterization. The methods used for characterization of helper cells such as Ig-positive and Ig-negative cell separation, removal of adherent and phagocytic cells, the use of anti-Thy-1.2 ascitic fluid, monospecific anti-Ly-1.1, and anti-Ly-2.1 antibodies, and complement treatments were essentially the same as described in earlier reports (25, 26). We are aware that Ly-1 antigen was recently demonstrated to be present in all T cells using very sensitive assay systems (32, 33), though quantitative difference may exist in various T cell subpopulations. However, in this report, the notations Ly-1⁺ and Ly-23⁺ cells are still used to denote cell populations that are sensitive to anti-Ly-1.1 and anti-Ly-2.1 antibodies, and complement treatment, respectively, under the prescribed experimental conditions.

Statistical Analysis. All results are expressed as the arithmetic mean \pm SE. Student's *t* test was used to determine the confidence limits in group comparisons.

Results

Evidence for Radioresistant Helper Cells for Primary DTH Response Generated to Influenza Virus In Vitro. Normal CBA spleen cells were incubated with A/WSN virus-infected syngeneic spleen cells (3 EID₅₀/cell) at a responder to stimulator cell ratio of 5:1. After 5 d of culture at 37°C, viable cells were tested for DTH activity by adoptive transfer with antigen (purified, uv-irradiated A/WSN virus) into syngeneic naive recipients. As shown in Table I, a low but significant level of DTH activity was generated without addition of other cells (group A). However, when spleen cells from mice primed in vivo 2 d earlier with a low dose of infectious A/WSN virus (10 HAU/mouse injected intravenously) were used as the responder cells, considerable enhancement of the DTH response was observed (Table I, group B). If without a further period of culture in vitro, these 2-d immune spleen cells were adoptively transferred intravenously to naive recipients or the Ig-negative fraction prepared from them was injected locally into the footpads of naive recipients and the mice were subsequently challenged with virus, little or no DTH activity could be detected (data not shown). On the other hand, if these 2-d immune spleen cells were cultured alone for 5 d in vitro and then injected into the footpads of mice with virus, a low but significant level of DTH activity was observed (Table I, group G). This suggests that sufficient antigen was carried over to allow the continuation of the in vivo-primed response in vitro. One possible explanation for the enhancement of the DTH response by in vivo priming was that the 2-d in vivo-primed cell population contained helper cells. If this was so, then this preparation would still be active after γ -irradiation. This was found to be the case. 2-d primed cells were γ -irradiated and added to infected stimulators and normal spleen cells (responders). An enhanced DTH response was shown when the cultured cells were adoptively transferred into naive recipients (Table I, group C).

TABLE I
Evidence for Radioresistant Helper Cells for Primary DTH Response Generated to Influenza Virus In Vitro

Group	Primary in vitro culture*				Percent mean increase in footpad thickness at 24 h§	Percent enhancement of DTH response
	A/WSN virus-infected spleen cells (1.5 × 10 ⁷)	2-d immune spleen cells‡ (7.5 × 10 ⁷)	2-d immune spleen cells (γ -irradiated, 2,000 rad) (7.5 × 10 ⁷)‡	Normal spleen cells (7.5 × 10 ⁷)		
A	+	-	-	+	15.6 ± 1.8	
B	+	+	-	-	34.4 ± 1.8	121
C	+	-	+	+	32.3 ± 2.8	107
D	+	+	-	+	32.3 ± 1.1	107
E	+	-	+	-	3.1 ± 0.0	—
F	-	-	+	+	10.2 ± 1.5	—
G	-	+	-	-	14.6 ± 1.1	—

* CBA mice were used as donors of spleen cells. Viable cells were harvested after culturing for 5 d. 1×10^7 cells were then injected together with purified, uv-irradiated A/WSN virus (6×10^3 HAU) into syngeneic mouse footpad. Footpad swelling was measured 24 h after challenge.

‡ Mice primed with 10 HAU infectious A/WSN virus.

§ Mean \pm SE for groups of 3-4 mice.

|| Percent enhancement was calculated as described in Materials and Methods.

If these γ -irradiated 2-d immune cells were cultured only with infected stimulator cells, no DTH activity was recovered (Table I, group E). This would argue against the possibility that the observed increase in DTH activity in group C was due to an additional DTH activity contributed by the primed cell population.

The next few experiments were carried out to determine optimum conditions for the production of this helper activity, and in all cases the helper cells were γ -irradiated before use.

Effect of In Vivo Priming Dose on the Generation of Helper Activity. CBA mice were primed intravenously with various doses (1–1,000 HAU) of infectious or uv-irradiated A/WSN virus and, 2 d later, spleen cells were harvested and used as the source of helper cells. Primary DTH activity was generated by the coculturing of γ -irradiated helper cells with A/WSN virus-infected spleen cells (stimulator cells) and normal spleen cells (responder cells) at a fixed ratio of 5:1:5. Viable cells were tested for their DTH activity 5 d later. As seen in Table II, significant levels of helper activity were generated by priming with low doses (1–10 HAU) of infectious virus; priming with higher doses of virus was less efficient. In contrast, if uv-irradiated virus was used for priming, doses of 10 HAU or higher were required to demonstrate significant levels of helper activity, and the helper activity was slightly increased at higher doses (e.g., up to 10^3 HAU).

Factors Affecting the Helper Activity for the DTH Response Generated In Vitro. In the first group of experiments, the kinetics of generation of helper activity were studied with the results shown in Fig. 1. 1-d immune spleen cells from mice primed with 10 HAU infectious virus were inactive, whereas optimal helper activity was obtained with 2-d immune spleen cells. Thereafter the helper activity declined gradually. About 40%

TABLE II
*Effect of In Vivo Priming Dose Using Infectious or Noninfectious Virus on the Generation of Helper Activity**

Virus dose used for priming	Infectivity of the virus used	Percent mean increase in footpad thickness at 24 h‡	Percent enhancement of DTH response
Experiment 1			
None	—	14.9 ± 1.5	—
1	Infectious	35.2 ± 0.8	136
10	Infectious	35.2 ± 1.5	136
100	Infectious	26.6 ± 2.0	79
1,000	Infectious	22.7 ± 2.7	52
Experiment 2			
None	—	13.6 ± 1.1	—
1	Noninfectious	17.7 ± 1.1	30
10	Noninfectious	32.3 ± 1.1	138
100	Noninfectious	34.4 ± 1.3	153
1,000	Noninfectious	39.9 ± 2.0	193

* CBA mice were primed intravenously with various doses of A/WSN virus. 2-d immune spleen cells were used as the source of helper cells. Primary DTH activity was generated by coculturing of γ -irradiated (2,000 rad) helper cells (7.5×10^7) with A/WSN virus-infected spleen cells (1.5×10^7) and normal spleen cells (7.5×10^7) for 5 d at 37°C.

‡ Primary effector cells (1×10^7) were injected into the footpad of each syngeneic recipient together with purified, uv-irradiated A/WSN virus (6×10^3 HAU), and footpad swelling was measured 24 h later.

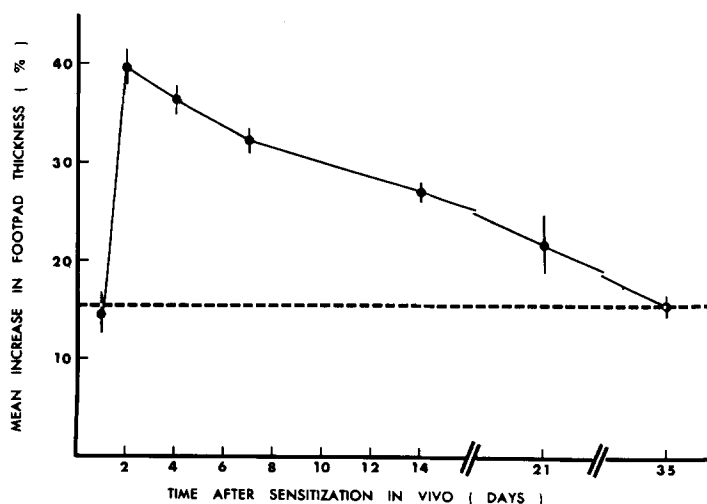


FIG. 1. Kinetics of generation of helper activity. CBA mice were primed at different intervals with 10 HAU infectious A/WSN virus injected intravenously. Normal or primed spleen cells were γ -irradiated and incubated with A/WSN virus-infected spleen cells and normal spleen cells at a ratio of 5:1:5. Cells were harvested on day 5 and tested for DTH activity by adoptive transfer (1×10^7 /mouse) with antigen (6×10^8 HAU purified, uv-irradiated A/WSN virus) into footpads of naive recipients. Vertical bars represent 1 SE.

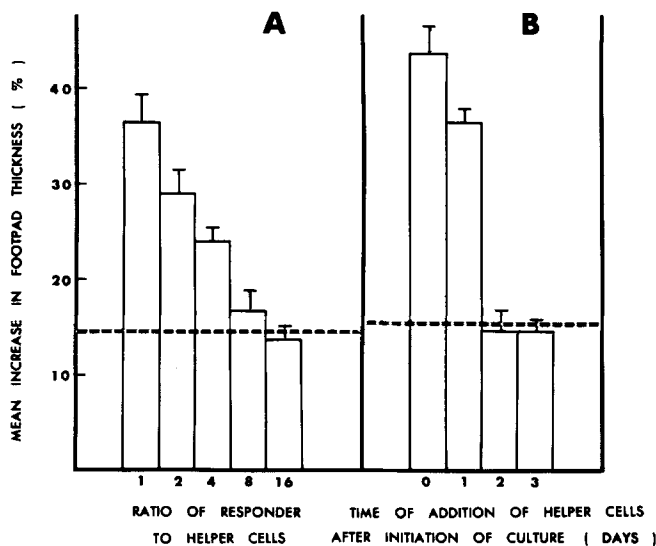


FIG. 2. (A) Dose-dependency of helper cells in augmenting primary DTH response generated to influenza virus in vitro. 2-d immune spleen cells from CBA mice primed with 10 HAU infectious A/WSN virus were added at different ratios with respect to normal responder cells. (B) Influence of the time of addition of helper cells in the enhancement of DTH response generated in vitro. 2-d immune spleen cells from CBA mice primed with 10 HAU infectious A/WSN virus were added at various times after initiation of primary culture. Ratio of helper, stimulator, and responder cells was always 5:1:5. Effector cells in (A) and (B) were harvested on day 5 and tested for DTH activity as described in Fig. 1. Horizontal lines indicate values of DTH activity from controls (no helper cells were added to cultures). Vertical bars represent 1 SE.

TABLE III
Helper Cell Characterization

Experiment	Source of helper cells*	Treatment of helper cells before culture‡	Percent mean increase in footpad thickness at 24 h	Percent enhancement of DTH response
1§	Normal spleen cells	None	17.7 ± 1.1	—
	2-d immune spleen cells	None	44.8 ± 2.8	153
	2-d immune spleen cells	Complement alone	45.8 ± 2.1	159
	2-d immune spleen cells	Anti-Thy-1.2 ascitic fluid + complement	16.7 ± 1.1	-6
2§	Normal spleen cells	None	14.1 ± 1.6	—
	2-d immune spleen cells	None	30.2 ± 1.1	114
	2-d immune spleen cells	Ig-positive fraction	12.5 ± 1.3	-11
	2-d immune spleen cells	Ig-negative fraction	34.4 ± 1.8	144
	2-d immune spleen cells	Removal of adherent cells	31.3 ± 0.0	122
3	BALB/c normal spleen cells	None	16.4 ± 2.4	—
	BALB/c 2-d immune spleen cells	None	39.6 ± 3.8	142
	BALB/c nude (<i>nu</i> ⁺ / <i>nu</i> ⁺) 2-d immune spleen cells	None	15.6 ± 3.1	-5
4§	Normal spleen cells	None	12.5 ± 0.0	—
	2-d immune spleen cells	None	42.7 ± 1.1	242
	2-d immune spleen cells	Anti-Ly-1.1 + complement	10.9 ± 0.9	-13
	2-d immune spleen cells	Anti-Ly-2.1 + complement	47.7 ± 1.9	282
	2-d immune spleen cells	Heat killed (56°C, 30 min)	14.6 ± 1.1	17
	2-d immune spleen cells	Sonicated and supernate used for culture	12.5 ± 1.8	0

* Normal spleen cells or cells from mice primed 2 d earlier with a low dose of infectious A/WSN virus (10 HAU, intravenously) were used as a source of helper cells.

‡ Helper cells were γ -irradiated (2,000 rad) in all cases (untreated or treated). 7.5×10^7 treated or untreated helper cells were cocultured with virus-infected stimulator cells (1.5×10^7) and normal responder cells (7.5×10^7) for 5 d at 37°C before harvesting for effector cells.

§ CBA mice were used.

|| Significantly higher than controls (no helper cells added) but not significantly different from each other for each experiment, ($P < 0.01$).

enhancement of the DTH response, as compared to control (normal spleen cells were used as helper), was still observed when 21-d immune cells were tested for helper activity, but 35-d cells were no longer active. In the second set of experiments, the dose-dependency of helper cells in augmenting primary DTH response generated in vitro to influenza virus was examined. The results in Fig. 2A clearly show that the helper activity was dose-dependent. Of the ratios of responders to helper cells tested, equal numbers of helper and normal responder cells gave maximal DTH response. No helper activity could be observed at a ratio of responder to helper cells of 8:1 or above. Finally, the influence of the time of addition of helper cells to the culture in the enhancement of DTH response generated in vitro was investigated. Fig. 2B shows that significant helper activity was observed if the cells were added within the first 24 h, but not 48 h or later, after the initiation of the culture.

Characterization of the Helper Cells. 2-d immune spleen cells from CBA mice primed with a low dose (10 HAU) of infectious A/WSN virus were treated with anti-Thy-1.2 ascitic fluid plus complement or with complement alone before their addition to cultures of stimulator cells and normal responder cells. Helper activity was completely abrogated by anti-Thy-1.2 ascitic fluid plus complement but not by complement treatment alone (Table III, experiment 1). Removal of plastic-adherent cells from the primed cell population did not affect their helper activity and fractionation of the resultant nonadherent cells into Ig-positive and Ig-negative fractions showed that only the Ig-negative cells contained helper activity (Table III, experiment 2), which suggests that the helper activity was a property of the T cells. Further confirmation of this was obtained by showing that nude mice failed to generate helper activity (Table III, experiment 3). The helper T cells were further characterized by anti-Ly

antibodies and complement treatment. It was found that the helper activity was sensitive to anti-Ly-1.1 antibodies plus complement, but not to anti-Ly-2.1 antibodies plus complement treatment (Table III, experiment 4). An experiment also showed that only viable cells could deliver help, because heat-killed cells or supernatant fluid from sonicated cells were unable to demonstrate any helper activity (Table III, experiment 4). Therefore, the data clearly show that radioresistant, viable plastic-nonadherent, Ly-1⁺ helper T cells can augment the primary DTH response generated to influenza virus in vitro.

Effects of Viral Infectivity and Antigen Specificity on T Cell Help. Four sets of experiments were carried out in which the infectivity of the virus used to generate the stimulator cells and helper T cells was varied. In addition, the effect of homologous or heterologous virus used to generate helper T cells was also examined. Normal CBA spleen cells (responders) were stimulated in vitro with syngeneic spleen cells exposed for 1 h

TABLE IV
Influence of Viral Infectivity on DTH Response Generated to Influenza Virus In Vitro

Experiment	Virus used					Percent mean increase in footpad thickness at 24 h	Percent enhancement of DTH response
	Priming mice for help*		Stimulating cells in vitro‡		Elicitation in recipients (uv-irradiated)§		
	Strain	Infectivity	Strain	Infectivity			
1	Control	—	A/WSN	NI	A/WSN (H0N1)	17.7 ± 1.1	—
	A/WSN	I¶	A/WSN	NI	A/WSN	42.2 ± 1.6**	138
	A/WSN	NI	A/WSN	NI	A/WSN	40.6 ± 3.1**	129
	Control	—	A/WSN	NI	Sendai	6.3 ± 1.3	—
	A/WSN	I	A/WSN	NI	Sendai	7.8 ± 1.6	24
	A/WSN	NI	A/WSN	NI	Sendai	3.1 ± 1.8	-50
	Control	—	A/WSN	NI	A/R.I. (H2N2)	6.3 ± 1.8	—
	A/WSN	I	A/WSN	NI	A/R.I.	4.7 ± 1.6	-25
	A/WSN	NI	A/WSN	NI	A/R.I.	5.2 ± 1.1	-18
	2	Control	—	A/WSN	I	A/WSN	19.8 ± 2.1
A/WSN		I	A/WSN	I	A/WSN	43.8 ± 3.6**	121
A/WSN		NI	A/WSN	I	A/WSN	41.7 ± 2.1**	111
Control		—	A/WSN	I	Sendai	6.3 ± 1.8	—
A/WSN		I	A/WSN	I	Sendai	6.3 ± 2.1	0
A/WSN		NI	A/WSN	I	Sendai	3.1 ± 0.0	-50
Control		—	A/WSN	I	A/R.I.	18.0 ± 1.5	—
A/WSN		I	A/WSN	I	A/R.I.	45.8 ± 1.1**	154
A/WSN		NI	A/WSN	I	A/R.I.	39.1 ± 1.6**	117
Control		—	A/WSN	I	A/P.C. (H3N2)	17.7 ± 1.1	—
A/WSN		I	A/WSN	I	A/P.C.	43.8 ± 1.8**	148
A/WSN		NI	A/WSN	I	A/P.C.	40.6 ± 1.8**	129

* CBA mice were primed with 10 HAU virus 2 d before harvest spleens for helper cells.

‡ Normal spleen cells exposed to either infectious (3 EID₅₀/cell) or purified, uv-irradiated (1.5 × 10³ HAU/10⁷ cells) A/WSN virus for 1 h at 37°C.

§ Purified, uv-irradiated virus (6 × 10³ HAU/mouse) was injected together with primary effector cells (1 × 10⁷/mouse) into footpads of syngeneic recipient mice.

|| Relative increase in footpad thickness compared to controls (mice received effector cells generated in the absence of helper cells).

¶ I, infectious; NI, noninfectious.

** Significantly higher than controls (no helper cells added) but not significantly different from each other, *P* < 0.01.

TABLE V
Specificity of Helper T Cells

Experiment	Virus used				Elicitation in recipients§ (uv-irradiated)	Percent mean increase in footpad thickness at 24 h	Percent enhancement of DTH response
	Priming mice for help*		Stimulating cells in vitro				
	Strain	Infectivity	Strain	Infectivity			
1	Control	—	A/WSN	NI	A/WSN	13.6 ± 1.1	—
	A/WSN (H0N1)	I¶	A/WSN	NI	A/WSN	32.8 ± 1.8**	141
	A/WSN	NI	A/WSN	NI	A/WSN	32.3 ± 1.1**	138
	B/LEE	I	A/WSN	NI	A/WSN	14.1 ± 1.6	4
	A/P.C. (H3N2)	I	A/WSN	NI	A/WSN	15.6 ± 1.8	15
	A/P.C.	NI	A/WSN	NI	A/WSN	14.6 ± 2.1	7
	A/JAP (H2N2)	I	A/WSN	NI	A/WSN	16.7 ± 3.8	23
	A/JAP	NI	A/WSN	NI	A/WSN	15.6 ± 1.8	15
2	Control	—	A/WSN	I	A/WSN	14.1 ± 1.6	—
	A/WSN	I	A/WSN	I	A/WSN	41.7 ± 2.1**	196
	A/WSN	NI	A/WSN	I	A/WSN	39.6 ± 2.1**	181
	B/LEE	I	A/WSN	I	A/WSN	16.7 ± 2.1	18
	A/P.C.	I	A/WSN	I	A/WSN	39.6 ± 2.8**	181
	A/P.C.	NI	A/WSN	I	A/WSN	35.4 ± 2.8**	151
	Control	—	A/WSN	I	A/P.C.	13.6 ± 2.8	—
	A/P.C.	I	A/WSN	I	A/P.C.	33.3 ± 1.1**	145
A/P.C.	NI	A/WSN	I	A/P.C.	33.3 ± 2.1**	145	

For explanation, see legend to Table IV.

at 37°C to either infectious (3 EID₅₀/cell) or purified, uv-irradiated virus (1.5×10^3 HAU/10⁷ cells) and the cultures also contained syngeneic γ -irradiated helper cells that were obtained from spleens of mice primed 2 d earlier with either homologous or heterologous virus and with infectious or uv-irradiated virus (10 HAU). Primary effector cells were harvested after 5 d and tested for their specificities of DTH activity by eliciting the reaction with different strains of purified, uv-irradiated virus (6×10^3 HAU). The results of several experiments are shown in Tables IV and V. As far as infectivity of the virus is concerned, the following two conclusions can be drawn: (a) if the virus used to generate stimulator cells in vitro is noninfectious, then the DTH effector cells so generated will be specific for the homologous virus used for stimulation, irrespective of whether infectious or noninfectious virus is used to generate helper T cells (Table IV, experiment 1); and (b) if the virus used to generate stimulator cells is infectious, then the final DTH effector cells will be cross-reactive within the A strains of influenza virus, again irrespective of whether infectious or noninfectious virus is used to generate helper cells (Table IV, experiment 2). That is, the specificity of the effector cells is predominantly a function of the infectivity of the virus used for their in vitro stimulation, and is independent of the infectivity of the virus used for in vivo priming for the helper activity. When the antigen specificity of the helper T cells was examined, the results showed that (a) helper activity is antigen-specific because helper cells generated from in vivo priming with type B influenza virus cannot help the primary response generated to type A virus (Table V); and (b) in vitro stimulation of DTH response by infectious virus can be helped by helper cells generated from in vivo priming with either homologous or heterologous A strain viruses, whereas

stimulation by noninfectious virus can only be helped by homologous, but not heterologous, A strain viruses (Table V).

H-2 Restriction of Helper Activity. γ -Irradiated helper cells were obtained from mouse strains that differed from the donors of the normal responder cells and of the virus-infected stimulator cells at various regions or subregions of the major histocompati-

TABLE VI
H-2 Restriction of Helper T Cells in Primary DTH Response to Influenza Virus Generated In Vitro

Donor mouse strains				Percent mean increase in footpad thickness at 24 h	
Helper cells (2-d immune spleen cells, γ -irradiated)	Normal responder cells and virus-infected stimulator cells	H-2 region shared by helper and responder cells	Injection into footpads of recipients*	Elicitation in	
				Recipients syngeneic to donors of responder cells using uv-irradiated virus \ddagger	Recipients K,D compatible to donors of responder cells using infectious virus \S
None	—	—	Virus only	3.1 \pm 2.1 \parallel	14.1 \pm 1.6 \parallel
Control	ATL	—	Cells + virus	14.1 \pm 1.6	23.4 \pm 1.6
ATL (s kkkkk kkd)	ATL	All	Cells + virus	34.4 \pm 0.0 \parallel (144)**	45.3 \pm 4.7 \parallel (94)**
C57BL/6J (b bbbbbb bbb)	ATL	Nil	Cells + virus	12.5 \pm 1.8 (-11)	23.9 \pm 1.1 (2)
CBA (k kkkkk kkk)	ATL	I	Cells + virus	32.3 \pm 1.1 \parallel (129)	41.7 \pm 1.1 \parallel (78)
B10.A (4R) (k kbbbbb bbb)	ATL	IA	Cells + virus	32.8 \pm 1.6 \parallel (133)	39.6 \pm 1.1 \parallel (69)
ATH (s sssss ssd)	ATL	K,D	Cells + virus	13.6 \pm 1.1 (-4)	23.9 \pm 2.1 (2)
BALB/c (d ddddd ddd)	ATL	D	Cells + virus	13.6 \pm 1.1 (-4)	22.9 \pm 1.1 (-2)

* Primary effector cells (5×10^6) were injected at the same time as uv-irradiated virus (6×10^3 HAU) or 6 h after injection of infectious virus (2.5×10^3 HAU).

\ddagger Only I-region restricted DTH activity could be elicited using uv-irradiated virus.

\S Only K,D-region restricted DTH activity could be demonstrated by elicitation with infectious virus in K,D-compatible recipients. In all cases, ATH mice were used as recipients except in the second to last group, where BALB/c mice were used to mimic any possible allogeneic effect of the cultured cells.

\parallel Nonspecific swelling due to injection of virus only.

\parallel Not significantly different from each other but significantly higher than the control group, (no helper cells were added), $P < 0.05$.

** Values in parentheses represent percent enhancement of DTH response as compared with those controls without addition of helper cells.

TABLE VII
H-2 Requirement for Delivery of Helper Activity

Donor mouse strains			H-2 region shared		Recipient mice strains (D-region compatible)	Injection into footpad of recipients \ddagger	Percent mean increase in footpad thickness at 24 h
Helper cells* (γ -irradiated)	Stimulator cells (γ -irradiated)	Responder cells	Helper vs. stimulator	Helper vs. responder			
None	—	—	—	—	BALB/c (ddd)	Virus only	14.1 \pm 0.9
Control	ATH	ATH	—	—	BALB/c	Cells + virus	25.0 \pm 0.0
ATH (ssd)	ATH	ATH	KID	KID	BALB/c	Cells + virus	39.9 \pm 2.4 \S
CBA (kkk)	ATH	ATH	—	—	BALB/c	Cells + virus	23.9 \pm 1.1
ATH	ATL (skd)	ATH	K,D	KID	BALB/c	Cells + virus	23.4 \pm 1.6
ATH	ATH	ATL	KID	K,D	BALB/c	Cells + virus	44.8 \pm 2.8 \S
Control	CBA	CBA	—	—	C3H0H	Cells + virus	23.9 \pm 1.1
CBA	CBA	CBA	KID	KID	C3H0H	Cells + virus	42.2 \pm 1.6 \S

* Both helper and responder cells were depleted of plastic adherent cells to minimize the possibility of cross-infection from stimulator cells, even though the latter were always extensively washed to remove any residual virus.

\ddagger 5×10^6 primary effector cells were injected 6 h after the injection of infectious A/WSN virus (2.5×10^3 HAU) into the same footpads.

\S Not significantly different from each other but significantly higher than controls (no helper cells were added), $P < 0.05$.

bility complex (MHC). After culturing for 5 d, the effector cells were adoptively transferred to naive recipients that were either syngeneic to the donors of the responder cells, when the challenge antigen used was uv-irradiated virus; or were K,D-region compatible but I-region incompatible with the donors of the responder cells when the challenge antigen was infectious virus. The results in Table VI show clearly that the helper cell activity was IA-subregion restricted, irrespective of whether the DTH effector cells generated were I- or K,D-region restricted.

In the above experiments, the stimulator and responder cells were syngeneic, so it was not clear whether the restriction occurred at the stimulator or responder cell level. Further experiments were carried out in which the helper cells were syngeneic at the I region, either with the stimulator cells or with the responder cells. The results in Table VII show that the I-region restriction of helper cell activity was at the level of stimulator cells and not with the responder cells.

Discussion

This paper shows that 2 d after intravenous injection of influenza virus into mice, the spleen contains T cells that can function as helper T cells (Th) in the in vitro generation of effector T cells that mediate DTH reactions. The cells are radioresistant, are sensitive to anti-Thy-1.2 ascitic fluid and complement and to anti-Ly-1.1 antibodies and complement treatment. Both inactivated and infectious virus induce Th cell formation but the dose response curves are different. With infectious virus, low doses were more efficient; with inactivated virus, higher doses were better. The reason for the different responses is not clear. It may in part be a question of antigen load. Although infectious virus has not been recovered from the spleen after intravenous injection of infectious virus (34), nonpermissive replication is thought to take place and this could result in a substantial increase in the amounts of antigen expressed at the cell surface. Although low levels of DTH activity can sometimes be detected in spleens 2 or 3 d after sensitization with virus, peak activity occurs at about day 6 (25). In contrast, peak Th activity occurs 2-3 d after sensitization and then slowly decreases and is no longer detected 5 wk later. In tissue culture, the Th cells are only effective if added to the culture within the 1st d but not 2 d after the initiation of culture. This is unlikely to be due to the fact that enough helper activity has been generated in the culture within the 1st 2 d as the lack of addition of Th cells usually gives only a low DTH response (Table I). It may be that the responder cells can no longer respond to the Th cells after 2 d in culture.

There are several aspects which warrant discussion. Although the Th cells show antigen specificity with respect to type A and B influenza viruses, the specificity pattern of T cell help and of the effector DTH cells depends both on the infectivity of the virus used for in vitro stimulation and on the specificity (i.e., homologous or heterologous virus within the A strains) of the virus used to generate the Th cells in vivo. The data, as shown in Tables IV and V, can be summarized as follows: (a) if infectious virus is used for in vitro stimulation, help is delivered whether homologous or heterologous virus is used to generate Th cells in vivo; (b) if noninfectious virus is used for in vitro stimulation, help is delivered only when homologous virus is used to generate Th cells in vivo. Infection of cells by virus may allow expression at the cell surface of a cross-reactive determinant(s) of a viral antigen, presumably the hemagglutinin (35, 36), and this may be recognized by the Th cells primed with a

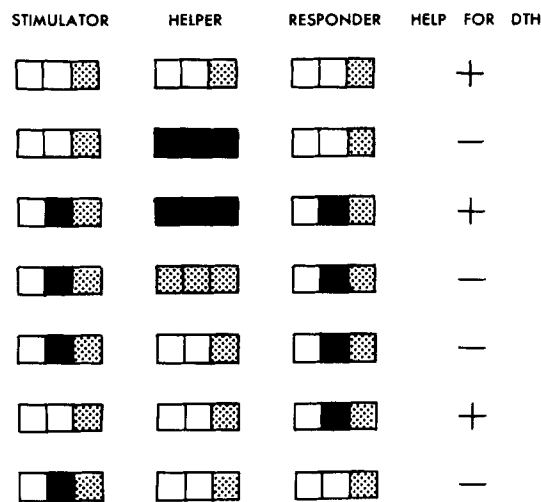


FIG. 3. Summary of H-2 restriction data. First panel, K region; second panel, I region; third panel, D region. □, s specificity; ■, k specificity; ▨, d specificity. The data show that the helper activity is I-region restricted and that this is directed to the stimulator and not to the responder cells.

heterologous virus. It has been shown that IgG response to influenza virus is T cell dependent (37), and both infectious virus and noninfectious virus are effective at inducing an antibody response after intravenous injection. A recent report (38) indicates that the Th cells present in the spleens of mice previously infected with an influenza A strain virus could deliver cross-reactive help (within the A strains) to B cells. It may be that the same Th cells can serve both roles, that is, to cooperate with both T and B cells.

Another interesting aspect is the H-2 restriction pattern of the Th cells and the main data for this, as recorded here, are summarized in Fig. 3. The activity of these cells is IA-subregion restricted, and by using stimulator and responder cells that varied in specificity at the K,D or I region with the helper cells, it was clearly shown that the restriction operates at the level of the stimulator cells and that the delivery of help to the responder cells is not H-2 restricted. There are many examples where it has been shown either *in vivo* or *in vitro* that Th cell activity is H-2 restricted (39-45). There are few *in vitro* experiments in which the H-2 requirements between Th cells and stimulator cells on one hand and Th cells and effector lymphocytes on the other hand have been studied. Singer et al. (43) showed that in a T-B collaboration system, the Th cells recognize H-2 determinants on accessory cells but not on B cells, so that the delivery of help to the B cells is not H-2 restricted. Ashman and Müllbacher (16) have also shown that the delivery of help by the radioresistant Th cells to the CTL precursors is H-2 unrestricted; the H-2 requirements between Th cells and stimulator cells was not analyzed.

It was found earlier that DTH activity to influenza virus can be mediated by two distinct subpopulations of T cells (29). One is Ly-2,3⁺ and is K,D-region restricted; the other is Ly-1⁺ and is IA-subregion restricted. The former is detected only if infectious virus is used both to sensitize the host and to elicit the response, and in this situation, both T cell subpopulations are demonstrated. If noninfectious virus is used at either step, only IA region-restricted DTH is observed. It was found that both the

K,D and IA region-restricted DTH reactions could be elicited from cell populations generated in the primary in vitro culture and the Th cells were active in augmenting the DTH activity mediated by both cell types. The question arises: what is the relationship between the precursor DTH cells, the effector DTH cells, and the helper cells? Recently, an interaction between two distinct T cell subpopulations, with the phenotypes Ly-123⁺ and Ly-1⁺ was shown to be necessary for the manifestation of the DTH response to a synthetic polypeptide (T,G)-A--L (24). We have shown elsewhere² that the precursors for primary DTH responses to influenza virus are most likely to be Ly-123⁺ cells, so that such precursors can give rise to two subpopulations of effector T cells. One is Ly-1⁺ and I-region restricted, and the other is Ly-23⁺ and K,D-region restricted. Presumably the helper T cells described in this work also arise from Ly-123⁺ precursor cells. Do they represent an intermediate stage between the precursor cells and the Ly-1⁺, IA subregion-restricted DTH effector cells? We do not know the answer to this question except to say that we know of no finding that eliminates this possibility. If this is so, then in the differentiation process, the ability to act as a Th cell precedes the ability to mediate a DTH response because the 2-d immune γ -irradiated cells are active as helpers but not as mediators of DTH reaction. Whatever the answer is, the present work adds to the growing evidence that provision of help via T cells is a common immunological phenomenon. The extent to which helper T cell activity in the generation of DTH response may occur in vivo has yet to be determined.

Summary

Injection of mice with infectious or noninfectious preparations of influenza virus induces the formation of T cells which, when added to primary tissue cultures of normal spleen cells exposed to influenza virus, enhance the generation of effector T cells which mediate delayed-type hypersensitivity (DTH) reaction. The enhancing cells possess Thy-1 and Ly-1 surface antigens are radioresistant and antigen-specific. If infectious virus was used to stimulate the DTH response in vitro, help was delivered whether homologous or heterologous A strain influenza virus was used to generate the helper T cells (Th) in vivo. In contrast, only Th cells generated using homologous virus were effective if noninfectious virus was used to stimulate the DTH response in vitro. Peak helper activity occurred 2 d after virus injection and the Th cells were only effective if added to the primary cultures within 24 h after addition of the stimulating antigen. The Th cells enhanced the generation of both classes of DTH effector cells, i.e., those that are Ly-1 positive and IA-subregion restricted and those that are Ly-2,3 positive and K,D-region restricted. The activity of the Th cells was found to be IA-subregion restricted and this was shown to operate at the level of the stimulator cells so that the delivery of help to the responder cells was not H-2 restricted. The possibility that the Th cells might be a precursor to the Ly-1 positive IA subregion-restricted DTH effector cells is discussed.

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