DELAYED-TYPE HYPERSENSITIVITY TO INFLUENZA VIRUS

Induction of Antigen-specific Suppressor T Cells for Delayed-Type Hypersensitivity to Hemagglutinin during Influenza Virus Infection in Mice

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Immune reponses are influenced by a network of interacting cells: regulatory amplification and suppression are often mediated by subsets of T cells. Suppressor T cells, both antigen specific and nonspecific, have been studied extensively in experimental systems for humoral and cell-mediated immunities (1–4). Suppressor cell activity has also been reported in several parasitic diseases (5–10). However, in most of the latter, only nonspecific suppressor cells have been demonstrated. We reported previously (11) that mice infected with influenza virus by aerosol or injected with purified ultraviolet (UV)[1] -inactivated virus or viral subunits developed weak and transient levels of delayed-type hypersensitivity (DTH) to influenza virus. The DTH reaction was greatly enhanced when mice were injected intraperitoneally with 150–200 mg/kg of cyclophosphamide (Cy) 2 d before immunization. The reaction was maximal 24–48 h after elicitation, had classical DTH histology, and was transferable by immune cells but not by immune serum. That pretreatment with Cy was necessary to induce significant and sustained levels of DTH suggests that suppressor cells for DTH may be preferentially induced during influenza virus infection (12–15). In this paper we report the detection of antigen-specific suppressor T cells for DTH to the viral hemagglutinin (HA) of influenza in virus-infected mice. This finding may explain why DTH has been difficult to detect during influenza infection. It may also lead to possible ways of modifying the host immune responses against this virus.

Materials and Methods

Mice. Inbred CBA/T6T6 mice, 10–12 wk old, of both sexes were used. They were bred and maintained at The Wellcome Research Laboratories, Beckenham, Kent, England. In all experiments, groups of five to six mice were used.

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[1] *Abbreviations used in this paper:* A/E/72, A/England/42/72; A/Pc/73, A/Port Chalmers/1/73; A/Scot/74, A/Scotland/840/74; A/Vic/75, A/Victoria/3/74; B/Lee, B/Lee/40; Cy, cyclophosphamide; DTH, delayed-type hypersensitivity; HA, hemagglutinin; HAI, HA inhibition; HRBC, horse erythrocytes; MID_{50}, mouse infective dose 50; PR8, A/Puerto Rico/8/34; Rec 31, A/England/939/69 × PR8; SRBC, sheep erythrocytes; TC, cytotoxic T-effector cells; TD, effector T cells for TDH; TH, T-helper cells for antibody response; TS, T-suppressor cells; TSD, T-suppressor cells for DTH; UV, ultraviolet; X31, A/Hong Kong/1/68 × PR8; X31 HA, bromelain-extracted monomer HA of X31 virus.

Preparation of Viruses and Viral Components. Viruses were grown in the allantoic cavity of 10-d-old embryonated fowl eggs. After incubation for 2–3 d at 37°C, allantoic fluids were removed, and the viruses were purified and concentrated by differential and sucrose density-gradient centrifugation. Incubation of purified viruses with bromelain (Sigma Chemical Co., London) released hemagglutinin as monomeric subunits and left residual spikeless viral particles. The monomeric hemagglutinin and the spikeless particles were separated and purified as described previously (11). Matrix protein was a gift from Dr. J. J. Skehel of the National Institute for Medical Research, London.

Treatment of Spleen Cells with Antisera and Complement. Aliquots of 1×10^8 spleen cells were resuspended in 1 ml of the various dilutions of antisera: anti-Thy-1.2 (F7D5 monoclonal IgM antibody, Olac Ltd., Oxon, England), 1/1,000; anti-Lyt-1.1 [(B6 × BALB/c)F_1 anti-B6-Ly-1^a], 1/6; anti-Lyt-2.1 [(B10.AKM × 129)F_1 anti-B6-PL (75NS)], 1/5; anti-Ia^k (A.TH anti-A.TL), 1/10. The amount of antiserum used was in excess for lysing cells of that particular specificity as judged by extrapolation from a standard cytotoxicity titration curve. After incubation for 30 min at room temperature, 10 ml of buffered salt solution containing 5% fetal calf serum was added and the cells were sedimented by centrifugation. The cells were resuspended in 2 ml of a 1/10 dilution of rabbit complement and incubated for a further 45 min at 37°C. At the end of incubation, the cells were washed twice and their viability estimated by trypan blue exclusion. Cytotoxicity of the various treatments was as follows: Anti-Thy-1.2 + complement (c′), 47.3%; anti-Lyt-1.1 + c′, 38.8%; anti-Lyt-2.1 + c′, 28.3%; anti-Ia^k + c′, 26.2%; c′ alone, 5%.

Assay for DTH. DTH was measured as footpad swelling. An eliciting dose of 10 μg of antigen in 50 μl phosphate-buffered saline was injected into the subcutaneous tissue of the right hind footpad. The increase in footpad thickness was measured 24 h later with a dial caliper (Pocotest, reverse spring-loaded caliper; Carobronze, England) that had graduations of 0.1 mm. DTH response was expressed as a percentage of footpad increase at 24 h.

The following virus strains were used in this study: A/England/939/69 × A/Puerto Rico/8/34 (Rec 31,) (H_3N_1); A/Hong Kong/1/68 × A/Puerto Rico/8/34 (X31) (H_3N_2); A/Puerto Rico/8/34 (PR8) (H_0N_1); A/England/42/72 (A/E/72) × PR8 (MRC2) (H_3N_2); A/Port Chalmers/1/73 (A/PC/73) × PR8 (MRC 11) (H_3N_2); A/Scotland/840/74 (A/Scot/74) (H_3N_2); A/Victoria/3/75 (A/Vic/74) (H_3N_2); B/Lee/40 (B/Lee).

Cy (cyclophosphamide monohydrate, Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England), was used at 150 mg/kg body weight 2 d before challenge injection. Parallel experiments using mice with or without Cy pretreatment yielded a similar degree of suppression by suppressor cells. However, we found it useful to pretreat mice with Cy before challenge to heighten the levels of DTH response to allow a higher degree of accuracy in the determination of suppression.

For infection, CBA/T_6T_6 mice were exposed to an aerosol of 10^2 mouse infective dose 50 (MID_{50}) of Rec 31 influenza virus.

Results

Effect of Influenza Virus Infection on DTH. In preliminary experiments, the effect of infecting mice with influenza virus on their subsequent DTH response to a challenge injection of purified, UV-inactivated virus was investigated. Table I demonstrates that mice infected with Rec 31 influenza virus by aerosol developed significantly lower levels of DTH to X31 influenza virus (which has a HA closely related to that of Rec 31) when compared to uninfected mice. These results contrast sharply with those of preimmunization with either (*a*) inactivated whole virus which has no effect on the subsequent DTH response to homologous virus[2] or (*b*) with matrix protein which potentiates both the humoral (16) and cell-mediated immunities[2] to the viral HA. Experiments were therefore designed to investigate this suppression further.

[2] Liew, F. Y., and C. M. Brand. T cells primed by influenza matrix protein can help in the delayed-type hypersensitivity to viral hemagglutinin. Manuscript submitted for publication.

TABLE I

*Effect of Influenza Infection on the Subsequent DTH Response to Inactivated Virus**

Mice	Immunization	DTH‡	Suppression§	P‖
		%	%	
Infected	10 μg X31 virus	18.3 ± 2.6	57.8	< 0.005
Normal	10 μg X31 virus	35.1 ± 3.1	0	—
Normal	—	6.0 ± 1.2	—	—

* Mice were infected with 10^2 MID_{50} of Rec 31 virus by aerosol. 2 wk later, these mice together with uninfected, normal mice were injected intraperitoneally with 150 mg/kg of Cy, and 2 d later, were immunized subcutaneously with 10 μg of purified, UV-inactivated X31 virus. These, together with control mice, were elicited for DTH in the footpad with 10 μg X31 virus. Footpad swelling was estimated 24 h after elicitation.

‡ DTH was expressed as the percentage of footpad thickness increase.

§ The percentage of suppression was calculated as:

$$\frac{(\text{DTH control} - \text{DTH background}) - (\text{DTH test} - \text{DTH control})}{\text{DTH control} - \text{DTH background}} \times 100.$$

‖ *P* value compared with control (line 2).

Adoptive Transfer of DTH Suppression. Spleen cells from mice infected 2 wk previously with Rec 31 virus were harvested and transferred intravenously into syngeneic recipients. The ability of these cells to suppress the induction or the expression of DTH to X31 virus in the recipients was tested with the following protocols. (*a*) Suppression of induction of DTH: recipient mice were injected intraperitoneally with 150 mg/kg of Cy on day 2. Donor cells were transferred on day 0. Immediately after cell transfer, recipients were immunized subcutaneously with 10 μg of purified, UV-inactivated X31 virus. DTH was elicited with X31 virus on day 6. (*b*) Suppression of expression of DTH: recipients were treated with Cy on day 8 and immunized subcutaneously with X31 virus on day 6. Donor cells were transferred on day 0. Immediately after cell transfer DTH was elicited with X31 virus. Table II shows that spleen cells from infected donors significantly suppressed the induction of DTH to X31 virus in normal mice (experiment A), whereas the same population of spleen cells failed to suppress the expression of DTH in mice sensitized to X31 virus before cell transfer (experiment B). Thus it appears that spleen cells from mice infected with influenza virus contain elements which suppressed the induction but not the expression of DTH to influenza virus antigens.

Fig. 1 depicts the results of a dose-response experiment. 1×10^8 or 5×10^7 spleen cells from Rec 31 virus infected donors suppressed the induction of DTH to X31 virus to a similar level, but 1×10^7 or 5×10^6 spleen cells had little or no effect on the DTH.

Effect of Treatment of Suppressive Spleen Cells with Antiserum and Complement. The cell type responsible for the suppressive effect was investigated by treating the donor spleen cells with various antisera directed against the cell surface phenotypic markers, and complement. Spleen cells from mice infected 2 wk before with Rec 31 virus were treated with a monoclonal anti-Thy-1.2 serum, anti-Lyt-1.1 serum, anti-Lyt-2.1 serum, or an anti-Ia^k serum and complement as described previously (17, 18).[2] After washing, the treated cells were transferred intravenously into syngeneic recipients which were pretreated with Cy. Control mice received either untreated donor cells or donor cells treated with complement alone, or normal spleen cells. Immediately after cell transfer,

TABLE II

*Effect of Transferring Spleen Cells from Mice Infected by Aerosol with Rec 31 Influenza Virus on the Induction and Expression of DTH to X31 Virus**

Experiment	Spleen cells from donors	Recipients	Immunization‡	Elicited§	DTH	Suppression	*P*
	1×10^8				%	%	
A	Infected with Rec 31	Normal	X31 virus	+	28.0 ± 1.6		
	Normal	Normal	X31 virus	+	43.0 ± 3.2	38.2	< 0.01
	—	Normal	—	+	3.7 ± 1.2		
B	Infected with Rec 31	Sensitized to X31 virus	—	+	44.9 ± 2.7		
	Normal	Sensitized to X31 virus	—	+	43.3 ± 5.2	−4.5	NS
	—	Normal	—	+	8.3 ± 1.3		

* For details see text and legend to Table I. NS, not significant.
‡ 10 μg UV-inactivated X31 virus subcutaneously.
§ 10 μg X31 virus in the footpad.

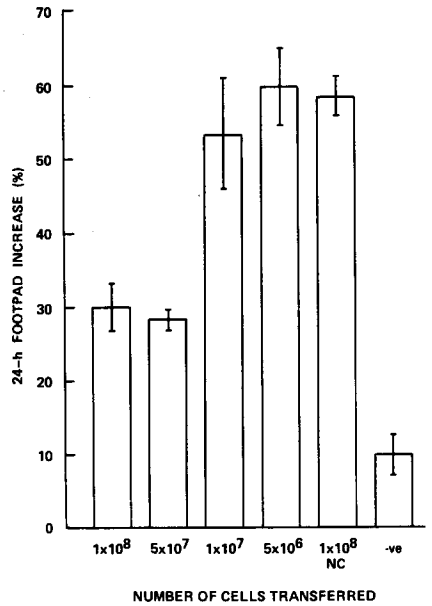

FIG. 1. Spleen cells from CBA mice infected 2 wk previously with Rec 31 virus were transferred intravenously at graded numbers into syngeneic recipients which were treated with Cy 2 d before cell transfer. Control mice received 1×10^8 normal spleen cells (NC). Immediately after cell transfer, recipients were challenged subcutaneously with 10 μg X31 virus. DTH was elicited with 10 μg X31 virus 6 d after challenge. "-ve" denotes background footpad increase in unchallenged mice.

recipients were immunized subcutaneously with 10 μg of X31 virus. 6 d later, DTH was elicited in the footpads with 10 μg of X31 virus. Results are depicted in Table III. The suppressive activity of the donor spleen cells was completely abrogated by treatment with anti-Thy-1.2 serum or anti-Lyt-1.1 serum plus complement. On the other hand, treatment with anti-Lyt-2.1 serum, anti-Ia^k serum plus complement, or complement alone had little or no effect on the suppressor activity. In a parallel

TABLE III

*Effect of Treatment of Suppressor Cells with Antiserum and Complement**

Donor spleen cells (1×10^8) treated with	DTH	Suppression	*P*
	%	%	
Untreated	30.0 ± 3.3	58.6	< 0.005
Anti-Thy-1.2 + c′‡	57.6 ± 3.5	1.5	NS
Anti-Lyt-1.1 + c′	47.6 ± 7.8	22.2	NS
Anti-Lyt-2.1 + c′	16.6 ± 2.8	86.4	< 0.001
Anti-Ia^k + c′	21.8 ± 1.4	75.6	< 0.002
Normal serum + c′	23.7 ± 4.5	71.7	< 0.005
Normal cells§	58.3 ± 2.7	0	—
Negative controls‖	10.0 ± 2.7	—	—

NS, not significant.

* For experimental details, see text.

‡ c′, complement.

§ Mice received normal untreated spleen cells.

‖ Negative controls denote background DTH. Normal mice not challenged but elicited only.

experiment, the antigen-nonspecific suppressor T cell for DTH induced in vivo by concanavalin A (Miles-Yeda Ltd., Israel) was abrogated by anti-Lyt-2.1 serum and complement but not by anti-Lyt-1.1 serum and complement (F. Y. Liew and I. F. C. McKenzie. Manuscript in preparation.) indicating that the lack of effect of the anti-Lyt-2.1 serum and complement in our experiment was not a result of an inactive serum. Thus, it appears that the suppression is mediated by T cells which are θ^+, Lyt-1^+2^-, and Ia^-.

Specificity of Suppressor Cells. The specificity of suppression of DTH by influenza virus infection was investigated by challenging Rec-31-infected mice with various antigens. Table IV shows that mice infected with Rec 31 virus showed a markedly reduced DTH response to the whole X31 virus. Their response to the bromelain-extracted monomer HA of X31 virus (X31 HA) was even more strongly suppressed. In contrast, although the DTH reaction to matrix protein appeared to be enhanced, the differences observed were not significant. These results are supported by the finding that DTH to the whole PR8 virus, which has similar internal components and neuraminidase to Rec 31 virus but differs in HA (H_0 subtype) was not affected. DTH reactions to B/Lee (an influenza B virus) and sheep erythrocytes (SRBC) were also unaffected in the Rec-31-virus infected mice. Thus it appears that the suppression of DTH by influenza infection is subtype specific, i.e., virus of the H_3 subtype (Rec 31) suppresses only the DTH to H_3 but not that of H_0 (PR8). It is of interest to compare the specificity of the T suppressor cells for DTH to that of the effector T cells for DTH which are also subtype specific but cross-react with the variants of the same subtype (11). Groups of mice were infected with Rec 31 virus and 2 wk later, they were challenged with inactivated preparations of the various influenza viruses of the H_3 subtype or of PR8 virus. 6 d after challenge, DTH was elicited with the homologous virus used for the challenge injection. Results in Table V demonstrate that the suppressor cells do not discriminate between the variants of the same subtype and thus appear to have the same level of specificity as that of T-effector cells for DTH.

TABLE IV

*Specificity of Suppression of DTH by Influenza Virus Infection**

Mice infected with Rec 31 virus	Challenge (subcutaneously)	Elicitation (footpad)	DTH	Suppression	*P*
			%	%	
+	10 μg X31 virus	10 μg X31 virus	20.7 ± 2.0		
−	10 μg X31 virus	10 μg X31 virus	29.3 ± 2.0	37.2	< 0.01
−	—	10 μg X31 virus	6.2 ± 1.0		
+	10 μg X31 HA	10 μg X31 HA	22.4 ± 1.4		
−	10 μg X31 HA	10 μg X31 HA	45.6 ± 3.1	68.5	< 0.005
−	—	10 μg X31 HA	11.7 ± 1.7		
+	10 μg MP	10 μg MP‡	43.8 ± 6.2		
−	10 μg MP	10 μg MP	37.0 ± 3.4	−16.4	NS
−	—	10 μg MP	2.3 ± 1.1		
+	10 μg PR8 virus	10 μg PR8 virus	27.3 ± 5.2		
−	10 μg PR8 virus	10 μg PR8 virus	25.6 ± 3.4	−8.4	NS
−	—	10 μg PR8 virus	5.5 ± 1.1		
+	10 μg B/Lee virus	10 μg B/Lee virus	46.3 ± 3.8		
−	10 μg B/Lee virus	10 μg B/Lee virus	49.6 ± 6.3	7.3	NS
−	—	10 μg B/Lee virus	4.3 ± 0.7		
+	1 × 10 SRBC	1 × 10 SRBC	68.2 ± 4.0		
−	1 × 10 SRBC	1 × 10 SRBC	68.7 ± 3.1	0.9	NS
−	—	1 × 10 SRBC	9.1 ± 0.9		

NS, not significant.

* CBA mice were either infected with an aerosol of Rec 31 virus or uninfected. 2 wk later, they were injected intraperitoneally with 150 mg/kg of Cy. 2 d after that, they were challenged subcutaneously with various antigens or remained uninjected as negative controls. 6 d after challenge, DTH was elicited with the corresponding challenge antigens.

‡ MP, matrix protein from A_2/Jap/305/57 × A0/Bel/42 (H_2N_1).

Although the data shown in Tables IV and V indicate that the suppression of DTH by Rec 31 virus infection was subtype specific, it could be argued that this specificity is in fact a reflection of specificity of antibody. Thus the infected mice produced specific antibody to the H_3 HA which could subsequently eliminate the challenge dose of X31 virus (H_3) resulting in reduced levels of stimulation of the effector-T cell population. To investigate this possibility, the specificity was tested by a cell transfer protocol. Spleen cells from mice infected with Rec 31 virus were transferred intravenously into syngeneic recipients which were challenged with X31 virus, PR8 virus, or SRBC. DTH was elicited 6 d later with the corresponding antigens. Again, only the DTH to X31 virus was significantly suppressed, DTH to all the other antigens was unaffected (Table VI). However, it can still be argued that the spleen cells transferred contained plasma cells that are capable of producing subtype-specific antibody in the recipients resulting in specific immunosuppression. To rule out this possibility, serum from infected donors were transferred into recipients. Table VII demonstrates that although spleen cells from Rec-31-virus infected donors substantially suppressed the

TABLE V

*Subtype Specificity of Suppression of DTH by Influenza Virus Infection**

Mice infected with Rec 31 virus	Challenged‡	Elicitation§	DTH	Suppression	*P*
			%	%	
+	X31 virus	X31 virus	27.2 ± 1.7		
−	X31 virus	X31 virus	41.1 ± 2.7	38.9	< 0.01
−	—	X31 virus	8.4 ± 0.8		
+	A/E/72 virus	A/E/72 virus	35.4 ± 1.5		
−	A/E/72 virus	A/E/72 virus	46.6 ± 1.6	27.7	< 0.01
−	—	A/E/72 virus	6.2 ± 1.0		
+	A/PC/73 virus	A/PC/73 virus	39.0 ± 3.3		
−	A/PC/73 virus	A/PC/73 virus	53.1 ± 1.3	28.1	< 0.02
−	—	A/PC/73 virus	3.0 ± 0.1		
+	A/Scot/74 virus	A/Scot/74 virus	32.9 ± 1.7		
−	A/Scot/74/virus	A/Scot/74 virus	48.8 ± 2.1	37.5	< 0.01
−	—	A/Scot/74 virus	6.4 ± 0.8		
+	A/Vic/75/virus	A/Vic/75 virus	42.3 ± 2.3		
−	A/Vic/75 virus	A/Vic/75 virus	61.3 ± 5.5	33.6	< 0.01
−	—	A/Vic/75 virus	4.8 ± 1.0		
+	PR8 virus	PR8 virus	44.7 ± 3.5		
−	PR8 virus	PR8 virus	45.9 ± 2.1	3.5	NS
−	—	PR8 virus	12.5 ± 1.4		

* For details see legend to Table IV. NS, not significant.
‡ 10 μg UV-inactivated X31 virus subcutaneously.
§ 10 μg X31 virus in the footpad.

DTH response to X31 virus, serum from the same donors failed to suppress the DTH reaction.

Kinetics of the Induction of DTH. The time-course of the induction of T-suppressor cells for DTH was also investigated. Mice were sacrificed at various times after infection with Rec 31 virus and spleen cells transferred into syngeneic recipients which were challenged with X31 virus. DTH was elicited with X31 virus after each challenge injection. Results in Table VIII show that suppressor cells were detectable 2 wk after infection and were present in the spleen for at least 40 d. By contrast, spleen cells from mice 7 d after infection significantly enhanced the DTH response in the recipients. The kinetic profile of the appearance of suppressor cells appears to be inversely related to that of DTH response of the donors (11). Mice infected with Rec 31 virus give a transient DTH reaction that peaks at day 6–7 after infection and no DTH is detectable thereafter (11). It seems likely that the enhanced DTH response in the recipients which received donor cells 7 d after infection may be a result of the transfer of T-effector cells rather than T-suppressor cells. Suppressor cells are therefore induced after the induction of T-effector cells and may account for the transient appearance of the DTH reaction in the donors. That transfer of spleen cells from

TABLE VI

*Specificity of Suppression of DTH by Influenza Virus Infection: Cell Transfer Study**

Donor mice infected with Rec 31	Recipients		DTH	Suppression	*P*
	Challenged (subcutaneously)	Elicited (footpad)			
			%	%	
+	X31 virus	X31 virus	31.9 ± 2.2		
−	X31 virus	X31 virus	56.3 ± 3.1	50.6	< 0.005
−	—	X31 virus	8.1 ± 0.9		
+	PR8 virus	PR8 virus	32.1 ± 2.8		
−	PR8 virus	PR8 virus	29.3 ± 3.7	−10.1	NS
−	—	PR8 virus	1.6 ± 1.6		
+	MP	MP	37.3 ± 4.7		
−	MP	MP	46.0 ± 1.2	20.2	NS
−	—	MP	3.0 ± 1.8		
+	SRBC	SRBC	42.2 ± 2.0		
−	SRBC	SRBC	48.4 ± 0.7	14.4	NS
−	—	SRBC	5.4 ± 1.1		

* Donor mice were infected with Rec 31 virus. 2 wk later, spleen cells were harvested and transferred intravenously (1×10^8 cells/mouse) into syngeneic recipients which were pretreated with Cy 2 d before cell transfer. Immediately after cell transfer, recipients were challenged with 10 μg of the viral antigens or 1×10^8 SRBC. 6 d later, DTH was elicited with 10 μg of viral antigens or 1×10^8 SRBC. NS, not significant.

TABLE VII

*Failure to Transfer Suppression of DTH by Antiserum**

Transfer (intravenous)	DTH	Suppression	*P*
	%	%	
1×10^8 primed spleen cells	19.1 ± 2.0	37.0	< 0.02
1×10^8 normal spleen cells	27.3 ± 2.3	−1.8	NS
0.5 ml immune serum	26.2 ± 1.8	3.3	NS
0.5 ml normal serum	24.1 ± 2.0	13.2	NS
—	26.9 ± 2.5	0	—
Negative controls	5.6 ± 0.7	—	—

* Donor mice were infected with Rec 31 virus. 2 wk later, spleen cells and serum were harvested and transferred intravenously into syngeneic recipients which were pretreated with Cy. Recipients were challenged subcutaneously with 10 μg of X31 virus immediately after transfer. DTH was elicited 6 d after challenge with 10 μg of X31 virus in the footpad. Negative controls denote background DTH in unimmunized mice. NS, not significant.

donors 1 wk after infection with Rec 31 virus leads to enhancement rather than suppression of DTH in the recipients also argues against the notion that the suppression of DTH in the recipients may be a result of the transfer of live virus via infected spleen cells. In our hands, mice infected with 10^2 MID_{50} of Rec 31 virus by aerosol produce a peak virus titer 2 d after infection, and little or no virus is detectable 7 d after infection (data not shown). Thus, if the transfer of infective virus was responsible

TABLE VIII

*Kinetics of the Induction of Suppressor Cells**

Spleen cells (1×10^8) from donors infected with Rec 31	Days after infection	Challenge	DTH	Suppression	P
			%	%	
+	7	+	42.7 ± 3.8	−359	< 0.001
−		+	16.0 ± 2.7		
−		−	5.7 ± 0.7		
+	14	+	9.1 ± 2.0	70.1	< 0.005
−		+	17.3 ± 2.3		
−		−	5.6 ± 0.7		
+	21	+	15.6 ± 0.0	47.2	< 0.01
−		+	22.2 ± 3.1		
−		−	8.2 ± 2.5		
+	40	+	21.0 ± 4.3	50.3	< 0.05
−		+	33.0 ± 3.1		
−		−	9.1 ± 1.1		

* At various times after infection with Rec 31 virus, donor spleen cells were harvested and transferred intravenously into syngeneic recipients which were challenged subcutaneously immediately with 10 μg X31 virus. DTH was elicited with 10 μg X31 virus 6 d after each challenge.

TABLE IX

TH to HA and TSD to HA are Coinduced

Donor spleen cells (1×10^8)	Recipients injected	DTH to X31 virus	HAI to PR8	
			Day 4	Day 7
		%		
Mice infected with Rec 31	10 μg X31 virus subcutaneously	31.9 ± 2.2*		
Normal mice	10 μg X31 virus subcutaneously	58.8 ± 1.8		
—	—	8.1 ± 0.9		
Mice infected with Rec 31	1 μg PR8 intraperitoneally		8.78 ± 0.19‡	8.18 ± 0.24§
Normal mice	1 μg PR8 intraperitoneally		6.38 ± 0.37	7.58 ± 0.31
—	1 μg PR8 intraperitoneally		6.58 ± 0	7.08 ± 0.28

* $P < 0.01$.
‡ $P < 0.001$.
§ $P < 0.05$.

for the DTH suppression, in a way similar to the nonspecific immunosuppression reported for measles virus infection in man (19), then one would expect the 7-d post-infection spleen cells to be at least as effective as the 40-d post-infection spleen cells in suppressing the DTH response in the recipients. This, in fact, was not the case.

T-Helper Cells for Antibody Repsonse (TH) and T-Supressor Cells for DTH (TSD) Are

Coinduced. In an earlier report (16), it was demonstrated that T cells primed by influenza virus internal components can cooperate in the antibody response to HA. It is therefore of interest to know whether TH are also activated under the condition where TSD are induced. Spleen cells from mice infected with Rec 31 virus were adoptively transferred into normal syngeneic recipients which were challenged intraperitoneally with PR8 virus. 4 and 7 d after challenge, recipients were bled and serum antibody against PR8 virus was determined by the hemagglutination inhibition (HAI) method. Data in Table IX show that the donor spleen cells suppressed the DTH response to X31 virus, but the same population of spleen cells potentiated the humoral response to PR8 virus. Because there is no cross-reaction between Rec 31 (H_3) virus and PR8 virus (H_0) the enhancement of the anti-PR8 antibody response indicates that TSD and TH may be coinduced in our system. Details for the activation and specificity of TH induced under the conditions presented in Table IX have been discussed previously (16).

Discussion

Our results strongly suggest that antigen-specific TSD for DTH, analogous to those induced by erythrocyte antigens (20, 21) and contact-sensitizing antigens (22–24) are generated during influenza virus infection. However, an alternative interpretation to our conclusion may be put forward. This is that cytotoxic T-effector cells (TC) that are generated during Rec 31 virus infection (25–28) may be eliminating virally modified stimulator cells. This form of suppression, which has been studied by Pang and Blanden (29) for TC against ectromelia virus may simply reflect the elimination of antigen-presenting cells necessary to generate effector populations operating in the DTH response to the challenge injection. This interpretation, however, seems less tenable in our system for the following reason. It is generally accepted that the TC belong to the subset bearing Lyt-$1^-2^+3^+$ phenotype (30–32). In our system, however, the suppressive activity was abrogated by anti-Lyt-1.1 serum plus complement but not by anti-Lyt-2.1 serum plus complement (Table III). This result argues strongly against the involvement of TC in the suppression of DTH.

The finding that TSD are Lyt-1^+2^- is of some interest. This is in direct contrast to the popular belief that suppressor cells are Lyt-$1^-2^+3^+$ (33, 34). However, the present data are in agreement with earlier results on the phenotype of suppressor cells for DTH. It was shown that cells responsible for the suppression of DTH to horse erythrocytes (HRBC) are Lyt-1^+2^- and Ia^-, and are different from cells that suppress the humoral response to HRBC, which are Lyt-1^-2^+ and Ia^+ (17). In yet another system, the production of suppressor factor for DTH to SRBC is dependent on Lyt-1^+ cells and not Lyt-2^+ cells (18). It is therefore possible that regulation of the various immune responses follows quite different pathways. Thus suppressor cells for the humoral response appear to be predominantly of the Lyt-1^-2^+ phenotype, whereas suppressor cells for DTH response are Lyt-1^+2^-, and the suppressor cells induced by graft-versus-host reaction are Lyt-$1^+2^+3^+$ (35, 36). On the other hand, there is increasing evidence that the immune response is regulated by a series of interactions between distinct T cell subsets. The cells, which initiate a series of events subsequently lead to immunosuppression, may in fact not be inhibitors but activators of certain active processes that are effectors of suppression. Thus in the humoral response to SRBC, Eardley et al. (37) proposed the circuit suppression hypothesis in which Lyt-

1^+2^- cells induce Lyt-$1^+2^+3^+$ cells to function as feedback suppressors. Other two-step mechanisms of T cell-mediated suppression of antibody synthesis have also been demonstrated for keyhole limpet hemocyanin-specific (38), and L-glutamic acid-L-tyrosine-specific (39) suppressor systems. Our results demonstrate that at least in one of the steps leading to the suppression of DTH in vivo, is dependent on a T cell subclass bearing the Lyt-1^+2^- surface phenotype. These results do not, however, define the sequence of events leading to the suppression of DTH induction.

In our system, TSD and TH are coinduced and carry the same surface phenotype. This raises the question whether TSD and TH are in fact, the same population of cells. Although there is no direct answer to this question, the following indirect evidence seems to suggest that they may be of different T cell subsets. As detailed elsewhere (16), TH in the present system are induced by the internal components of the virus and are matrix protein from A_2/Japan/305/57 × A_0/Belgium/42 virus specific (Table IX: X31 virus and PR8 virus have similar matrix proteins but non-cross-reacting HA). TH potentiate the HA response by way of associative recognition of matrix protein and HA in the intact virus, analogous to the carrier-hapten system.[3] In contrast, TSD appear to be induced by the HA of influenza virus and are specific for HA, having little or no effect on the DTH to MP (Tables IV and VI). Thus, even if TH and TSD are of the same cell type, they would be of different specificities. On the other hand, it could be argued that the suppression of DTH is in fact mediated by TH by means of antibody feedback mechanism (40). Thus, TH upon transferred to syngeneic recipients potentiate the antibody response to HA which could subsequently suppress the DTH response. However, this interpretation seems unlikely, as it will be shown below that antibody does not appear to play an important role in the suppression of DTH in the present system.

TSD for influenza virus appear to be subtype specific but make no distinction among the variants of a subtype. Their specificity is therefore of the same level as that of the effector T cells for DTH (TD) which are also subtype specific but do not discriminate between the antigenic variants of influenza virus (11). The specificity of DTH suppression in our study is not likely to be mediated by humoral antibody for the following reasons: (*a*) The variants of a particular subtype are classified and distinguishable by humoral antibody. Thus, if the DTH suppression is mediated by antibody, one would expect the levels of suppression to be dissimilar for the various viruses and the degree of suppression would be in direct correlation to the degree of serological cross-reaction between the variants. However, the level of DTH suppression affected by Rec 31 virus infection on the various influenza viruses of the H_3N_2 series are similar (Table V). (*b*) Serum from infected donors fails to suppress the DTH response, whereas spleen cells from the same donors do (Table VII).

It is interesting to note that TSD seem to appear after TD during the influenza virus infection. This may reflect the difference in relative antigenic doses required for the induction of TD and TSD. It has been demonstrated that TD require relatively lower dose of antigens for induction whereas higher doses of antigen are necessary for the stimulation of the precursors of TSD (21–24). Thus, earlier in the course of influenza infection when the antigen load is lower, TD are preferentially induced,

[3] Russell, S. M., and F. Y. Liew. Cell cooperation in antibody responses to influenza virus. I. Priming of T helper cells by internal components of the virion. Manuscript submitted for publication.

however, as the infection reaches the peak level, the higher load of virus in the host would induce TSD rather than TD. Such an interpretation could account for the DTH response profile during influenza infection (11). It also implies that TD are relatively short-lived and are replenished continuously, because the TSD inhibits only the induction of DTH and not the expression of DTH (Table II). It is now clear that suppressor T cells for the induction of DTH and the suppressor T cells for the expression of DTH are distinct and may be separated into different cell clones (41). The reason why only TSD for induction are activated is still unclear. The late and sustained presence of T suppressor cells appears to be characteristic of infectious diseases. During *Leshimania tropica* infection in BALB/c mice for instance, TD appear before TSD which are detectable long after initiation of infection and responsible for the specific suppression of TD to the parasite (J. G. Howard, C. Hale, and F. Y. Liew. Manuscript in preparation). This is in contrast to experimental systems using high doses of artificial antigens in vivo, where T suppressor cells appear 2–3 d after antigen administration and are not detectable in lymphoid organs 5–6 d later (21, 42–44). This apparent discrepancy can readily be accounted for by considering the replicating nature of the parasite and the persistent presence of parasitic antigens which maintain a continuous stimulation of TSD.

In an earlier report we have shown that T-helper cells (TH) for antibody response to HA are induced by matrix protein (16), and matrix protein is shown to be able also to induce TH cells for DTH (THD) to HA.[2] However, in this paper, the T-suppressor cells for DTH directed against HA have no apparent effect on the DTH to matrix protein. It has been proposed that suppressor T cells and TH differ in their functional specificity repertoires (45) such that certain determinants on a macromolecule are helper epitopes whereas others may be suppressor epitopes. If this is so, one could postulate that functional specificity repertoires are also distinct among subsets of suppressor T cells (such as TSD and suppressor T cells for antibody response), because T suppressor cells in our system appear to be specific for DTH, and the antibody response to HA in influenza-infected mice is augmented (Table IX), indicating that T-helper cells rather than T-suppressor cells are amplified.

It has been shown that priming with the whole virus has no effect on the subsequent DTH response to HA.[2] This is interpreted as being the result of an antagonistic interaction between the helper cells induced by matrix protein and the suppressor cells induced by HA of the whole virus. The existence of an epitope on antigenic molecules able to activate suppressor T cells (TS) to nullify the positive effect induced by TH reactive with other epitopes has been demonstrated in the response to β-galactosidase (46), bovine serum albumin (47), lysozymes (48), and in the myelin basic protein system (49). Myelin basic protein, which can induce experimental allergic encephalomyelitis, can be cleaved into distinct regions, one of which can cause the encephalitis, whereas a different one can specifically induce TS to prevent the disease upon subsequent challenge with myelin basic protein. Our results with the DTH response to the influenza virion antigens are, therefore, akin to the above examples for the humoral responses.

It is necessary to account for the difference between the suppression of DTH during influenza infection and the apparent lack of DTH suppression by immunization with inactivated influenza virus. The answer may be found in the different mode of antigen presentation by the different components of the virion during virus replication in the host. Inactivated viruses when administered to the host may passively attach to the

host cells, such as macrophages, via their HA, thus providing equal presentation probability to both the suppressor epitope (HA) and the helper epitope (matrix protein). In contrast, replicating influenza viruses express mainly viral subunits such as HA on the surface of infected cells and internal components such as matrix protein are barely detectable on the surface of host cells (50–52). Thus, there may be presentation advantage for the suppressor epitope (HA) leading to the predominant activation of TSD by HA.

Our findings therefore suggest that influenza virus, by virtue of its well-characterized antigens, may be a useful tool in studying the basic mechanism of immunoregulation in infectious diseases. It would be of interest to know whether the recent finding of the apparent difference in susceptibility to influenza virus between various congenic strains of mice (53) could be accounted for by the major histocompatibility complex-controlled activation of different T cell subpopulation (54) by the same or different epitopes of the viral antigen.

The finding that antigen-specific suppressor cells for DTH to HA occur during influenza virus infection may have practical implications. It readily explains the low level of DTH induced during influenza virus infection. Our initial results suggest that DTH to influenza virus, when induced, may have a protective role against the virus infection. This is particularly interesting as DTH appears to have broader cross-reactivity compared to antibody response (11). The identification of specific suppressor T cells in this system suggests that methods of immunization that would circumvent their induction might be of advantage.

Summary

Mice infected with A/England/939/69 × A/Puerto Rico/8/34 (Rec 31) influenza virus by aerosol develop significantly lower levels of delayed-type hypersensitivity (DTH) to A/Hong Kong/1/68 × A/Puerto Rico/8/34 (X31) virus compared to uninfected mice. The suppression of DTH to the hemagglutinin appears to be mediated by suppressor T cells which carry Lyt-1 membrane antigen marker, and not by serum antibody. The suppressor T cells for DTH induced by Rec 31 virus (H_3N_1) infection suppress the DTH response to the variants of the H_3 subtype of influenza viruses, but have no effect on the DTH responses to A/Puerto Rico/8/34 virus (H_0N_1), a B influenza virus or the matrix protein of type A influenza virus. Suppressor T cells for DTH appear 2 wk after infection and are detectable in the spleen for at least 40 d thereafter. T-helper cells for antibody response to hemagglutinin are induced concomitantly with the T-suppressor cells for DTH. Possible implications of the present findings on the regulation of the immune response to viral infection are discussed.

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