

## VIRUS-REPLICATING T CELLS IN THE IMMUNE RESPONSE OF MICE\*

### I. Virus Plaque Assay of the Lymphocytes Reactive to Sheep Erythrocytes

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Thymus-derived lymphocytes (T cells) play central roles in immune responses. They include effector cells in cell-mediated immune responses such as delayed-type hypersensitivity (DTH),<sup>1</sup> allograft rejection, tumor immunity and cellular resistance to infection, mediate helper function in antibody production, and act as suppressor cells in both cell-mediated and humoral immune responses (1, 2). At present these T-cell-mediated functions are studied with various functional assay systems such as killing of target cells (3), production of lymphokines (4), proliferative T-cell response (5), or carrier effect in antibody production (6). These assay systems contributed much in the elucidation of the nature of T-cell-mediated functions. However, all of these methods are restricted to measure the activities of a certain cell population. The development of more quantitative methods has been desired which would enable us to enumerate antigen-specific T cells at the single cell level analogous to Jerne plaque assay for antibody-producing cells.

Bloom et al. (7) introduced a hopeful method to enumerate activated T cells. They showed that the antigen-reactive lymphocytes became capable of replicating vesicular stomatitis virus (VSV) when lymph node cells from guinea pigs showing DTH to tuberculin were cultured in vitro with purified protein derivative of tuberculin (PPD). As VSV-replicating cells were not observed in the culture of lymph node cells taken from the animals that were immunized so as to produce antibody against PPD without accompanying DTH, it was strongly suggested that antigen-induced virus plaque-forming cells (V-PFC) represented the lymphocytes involved in DTH. Subsequently, Kano et al. (8, 9) showed that V-PFC were generated in the mitogen-stimulated culture of normal mouse lymphocytes or in the mixed lymphocyte culture, and that these V-PFC belonged to T cells. Thus, it has been expected that this new method might be useful as a tool for the enumeration of antigen-reactive T cells. However, there still remain at least two problems for virus plaque assay before it can be used generally. The first one is whether antigen-induced V-PFC can be detected in the immune responses to any other antigens, especially to those widely used in the study of both humoral and cell-mediated immune responses. The other is whether virus plaque assay measures all the activated T lympho-

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<sup>1</sup> *Abbreviations used in this paper:* Ag-V-PFC, antigen-stimulated virus plaque-forming cells; AS4, absorbed anti-sheep red blood cell antiserum; ATS, rabbit anti-mouse thymocyte serum; CY, cyclophosphamide; DTH, delayed-type hypersensitivity; HRBC, horse red blood cells; PFC, plaque-forming cells; US4, unabsorbed anti-sheep red blood cell antiserum; VSV, vesicular stomatitis virus.

cytes or only a subpopulation of T lymphocytes, such as effector cells, helper cells, or other cells involved in the regulation of immune responses.

In the present study, we attempted to apply virus plaque assay to analyze the immune response of mice against the widely used antigen, sheep red blood cells (SRBC), since in this experimental system we would most easily be able to deal with both humoral and cell-mediated immune responses simultaneously. T lymphocytes which acquired the capacity to produce VSV by antigenic stimulation (Ag-V-PFC) were generated in the culture of spleen cells from mice that had been primed with SRBC. These Ag-V-PFC detected in our experimental system did not seem to represent the helper cells nor the effector cells mediating DTH, but seemed to represent SRBC-reactive T cells involved in the regulation of DTH.

### Materials and Methods

*Animals and Immunization.* Male CBA/StMs mice originally obtained from National Institute of Genetics, Mishima, Japan, aged 8- to 10-weeks old, were used throughout the experiments. Mice were immunized with SRBC by intravenous injection via the tail vein.

*Conjugation of 2,4,6-Trinitrophenol (TNP) to Erythrocytes.* Heavily conjugated TNP-SRBC was prepared according to Kettman and Dutton (10) for in vitro assay of carrier effect. The preparation of TNP-conjugated horse red blood cells (TNP-HRBC) for the assay of anti-TNP plaque-forming cells was essentially the same as that reported by Rittenberg and Pratt (11).

*Cell Suspensions and Culture.* Spleen cell suspensions were prepared as described previously (12). After washings, the cells were resuspended in Eagle's minimal essential medium (MEM) (Nissui Seiyaku, Japan) supplemented with 8% heat-inactivated fetal calf serum (Flow Laboratories, Inc., Rockville, Md.), and  $5 \times 10^{-5}$  M 2-mercaptoethanol. The cell concentration was adjusted to  $1.2 \times 10^7$  viable cells per ml, and 1.5 ml of the cells was cultured per dish. For virus plaque assay, the cells were cultured with  $1 \times 10^7$  SRBC in 35-mm plastic dishes (Falcon Plastics, Oxnard, Calif.). In the experiment to test the cross-reactivity,  $1 \times 10^7$  HRBC were also used as the in vitro antigen. For the assay of helper T-cell activity, the cells were cultured with  $1 \times 10^7$  heavily conjugated TNP-SRBC. In all experiments, control cultures were also set up parallelly in antigen-free medium. They were incubated at 37°C in 5% CO<sub>2</sub> in humidified air.

*Antisera.* Mouse anti-SRBC serum was obtained from mice given intravenous injection of  $10^9$  SRBC 4 days previously and heated at 56°C for 30 min. This unabsorbed serum (hemagglutinin titer 1:1,024) was called US4 according to Mackaness et al. (13). The US4 was absorbed three times with equal volume of packed SRBC. This absorbed serum was called AS4, the hemagglutinin titer of which was lower than 1:2.

Rabbit anti-mouse thymocyte serum (ATS) was prepared as described previously (14). After the incubation of cells (final  $10^7$  per ml) with ATS (final 1:100) and guinea pig serum (final 1:10) at 37°C for 45 min, more than 95% of the thymocytes, 37-53% of the spleen cells, and less than 5% of the bone marrow cells were killed. Even with 1:5 dilution of ATS, killing of bone marrow cells did not increase.

*Assay of Antibody-Producing Cells.* Antibody-forming cells were assayed as PFC in agarose gel on microslides (15). Anti-TNP antibody-forming cells were assayed using TNP-HRBC. Anti-mouse immunoglobulin rabbit serum (1:200) was used to develop indirect PFC.

*Test for DTH.* Procedures of the injection of test antigen into the footpads and the measurement of the swelling were essentially the same as those used in the previous study on DTH to bovine serum albumin (14). DTH to SRBC was estimated by the swelling of one footpad 24 h after the injection of  $1 \times 10^8$  SRBC. In one experiment, an eliciting dose of HRBC was injected into one footpad to check the cross-reactivity.

#### *Virus Plaque Assay*

**VIRUS.** VSV, New Jersey strain, was supplied from National Institute of Animal Health, Tokyo, Japan. They were grown in L cells and stored at -70°C after centrifugation to remove cell

debris. After thawing, virus was titrated by plaque technique and expressed as plaque-forming units (PFU).

**L CELLS.** L-158 cells were maintained continuously in Roux bottles in MEM supplemented with 5% calf serum (CS). For the virus plaque assay,  $1 \times 10^6$  cells in 2 ml of MEM containing 5% CS were plated in 35-mm plastic dishes 24 h before use, and incubated at 37°C in 5% CO<sub>2</sub> in air. At the time of assay, the plates used had a confluent monolayer of L cells.

**ANTI-VSV SERUM.** Rabbit anti-VSV serum was kindly supplied by Dr. G. Tokuda (National Institute of Animal Health). The serum was inactivated at 56°C for 30 min, filtered through Millipore filter, and stored at -20°C. The serum (final 1:10) was found to be able to neutralize at least  $1 \times 10^8$  PFU of VSV.

**VIRUS PLAQUE ASSAY.** Cultured spleen cells were washed, resuspended in MEM, and adjusted to  $2-3 \times 10^6$  cells per 0.2 ml. 0.2 ml of cell suspensions were infected with VSV and incubated at 37°C in 5% CO<sub>2</sub> in air for 2 h. The multiplicity of infection was about 30. Excess viruses were removed by two washings, and the remaining free viruses were neutralized by incubating the cell suspensions (0.5 ml) with 0.05 ml of anti-VSV serum at 4°C for 1 h. After three further washings, cell suspensions were adjusted to  $1 \times 10^5$  cells per ml. The medium of the monolayers of L cells were drained, and 0.1 ml of VSV-infected cells ( $1 \times 10^4$ ) were added on the monolayers. Then 0.4 ml of 1% agar in MEM containing 6% CS, maintained at 42-44°C, was poured and mixed gently with the cells. After this layer of agar had solidified, another layer of 1 ml warm agar-MEM was added as a nutrient layer. Each cell sample was plated in triplicate. Samples of the supernate of the final centrifugation of VSV-infected lymphocytes were also plated. Since these supernatant samples did not contain viruses, virus plaques appearing in the L-cell monolayers were regarded as plaques by virus replicated in cells. The dishes were incubated at 37°C in 5% CO<sub>2</sub> in air for 2 days, and then vitally stained with neutral red (1:10,000) in phosphate-buffered saline.

**CALCULATION OF AG-V-PFC.** Since there existed inevitable background V-PFC in the control cultures (1,000-4,000/ $10^6$  plated cells), we determined Ag-V-PFC as follows according to Kano et al. (9).  $\text{Ag-V-PFC} = (\text{V-PFC in the antigen-stimulated culture}) - (\text{V-PFC in the control culture without antigen})$ . Thus, Ag-V-PFC are cells that newly acquired the capacity for replicating VSV by antigenic stimulation. It was shown by Nowakowski et al. (16) that almost all of the background V-PFC were macrophages.

## Results

*Generation of V-PFC in the Culture of Spleen Cells from Mice Primed with SRBC.* Spleen cells from normal mice or mice primed 5 days previously with varying doses ( $10^5$ ,  $10^7$ , or  $10^9$ ) of SRBC were cultured with or without SRBC. Cultured cells were harvested 1, 2, or 3, days later, and virus-replicating cells were enumerated on L-cell monolayers. The results of the generation of Ag-V-PFC in the culture are shown in Fig. 1 with solid lines. Each point in the figure represents the mean Ag-V-PFC of 2-6 separate experiments. In each experiment, two cultures were pooled, and a triplicate plating for virus plaque assay was performed. In normal, unprimed spleen cells, a significant number of Ag-V-PFC was not generated during 3 days in the culture (Fig. 1 A). Increase in V-PFC by antigen stimulation was seen in the cultures of cells from primed mice, while the kinetic patterns of generation differed in relation to the immunizing doses. In the cultures of spleen cells from mice primed with relatively low doses of SRBC ( $10^5$  or  $10^7$ ), the number of Ag-V-PFC increased for 2 days, and this was followed by a rather abrupt decrease (Fig. 1 B and C). By contrast, the number of Ag-V-PFC in the spleen cell culture of mice primed with a high dose of SRBC ( $10^9$ ) was maximal on the 1st day and gradually declined thereafter (Fig. 1 D).

In the previous paper (17), it was shown that the kinetics of generation of Ag-V-PFC in the culture was quite different from that of anti-SRBC PFC. In the present experiments, generation of Ag-V-PFC was compared with that of helper

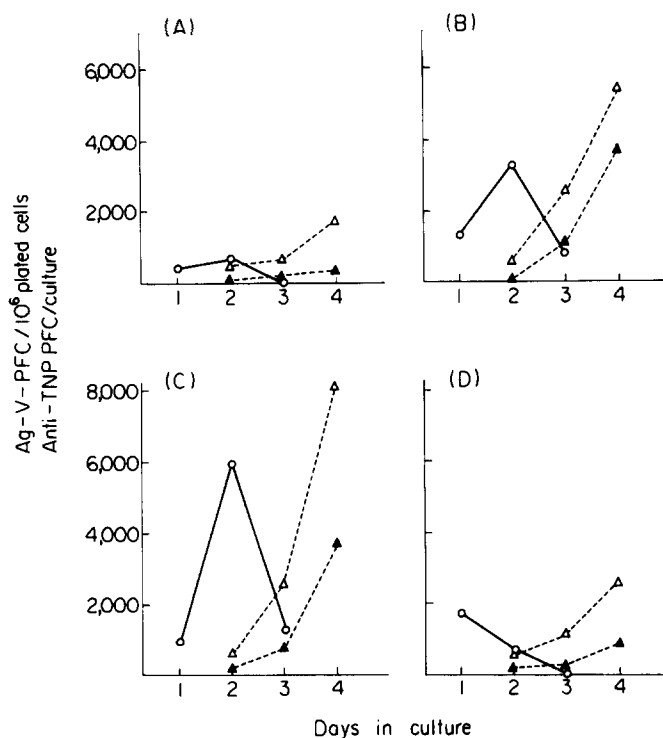


FIG. 1. Kinetics of the generation of Ag-V-PFC and anti-TNP PFC in the culture of spleen cells. Spleen cells from normal mice (A), or mice primed with  $10^5$  (B),  $10^7$  (C), or  $10^9$  (D) of SRBC were cultured with or without the addition of SRBC for virus plaque assay, and with heavily conjugated TNP-SRBC for helper assay. Virus plaque assay was performed on day 1, 2, and 3 of culture, and the numbers of Ag-V-PFC (○—○) were calculated as described in Materials and Methods. Each point represents arithmetic mean value of Ag-V-PFC from two to six separate experiments. For each experiment, a triplicate plating was performed. Direct (△---△) and indirect (▲---▲) anti-TNP PFC were assessed on day 2, 3, and 4 of culture of the spleen cells taken from the same mice as used in one of the virus plaque assays. Each point represents the arithmetic mean value of three cultures.

T-cell activity. Spleen cells taken from the same mice used in one of the V-PFC experiments were cultured with or without the addition of heavily conjugated TNP-SRBC. Anti-TNP PFC response was assayed on day 2, 3, or 4. As shown in Fig. 1 with broken lines, helper activity was induced significantly by priming with relatively low doses of SRBC ( $10^5$  or  $10^7$ ) rather than with high dose ( $10^9$ ), conforming to the data reported by Falkoff et al. (18). According to Lagrange et al. (19), DTH against SRBC was induced by intravenous immunization in which the immunization with lower doses of SRBC ( $10^5$  or  $10^7$ ) was more effective in producing DTH than the high dose ( $10^9$ ). Similar results were also obtained by us in CBA mice (data not shown). Thus, as far as the requirement of the priming dose is concerned, the generation of Ag-V-PFC seems to correlate with both helper activity and DTH.

*ATS Sensitivity of Ag-V-PFC.* In the preceding publication, we showed that the treatment of primed spleen cells before culture with ATS (1:100) plus guinea pig serum (C') completely abolished (about 97%) the generation of Ag-V-PFC

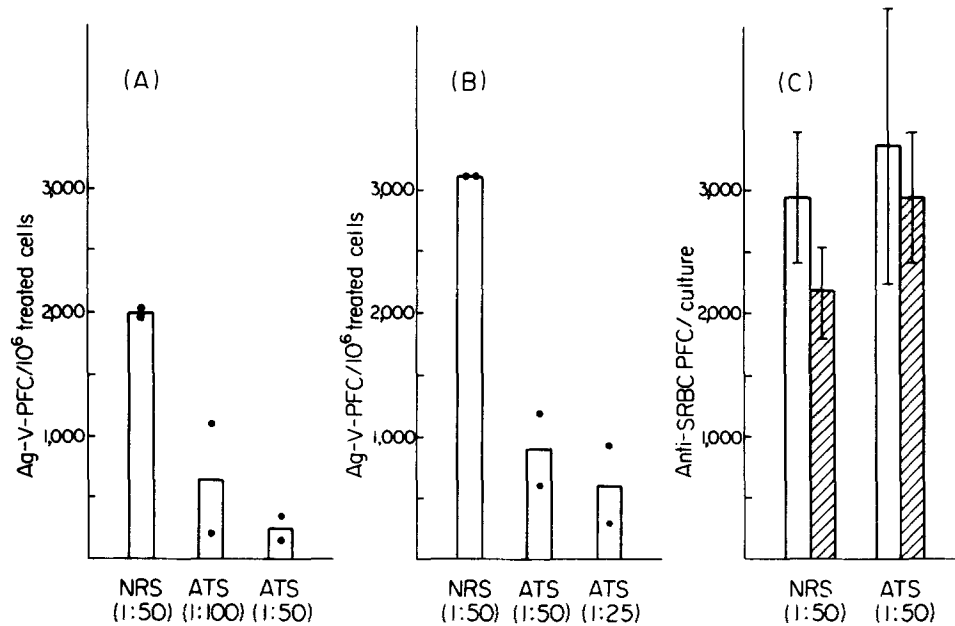


FIG. 2. Abrogation of Ag-V-PFC by the treatment of cultured cells with ATS and guinea pig serum (C'). Spleen cells from mice primed with  $10^7$  SRBC 4 days (A) or 5 days (B) previously were cultured with or without SRBC for 2 days. The cultured cells were infected with VSV, and  $10^7$  of infected cells were treated with ATS (final 1:100-1:25) or NRS (final 1:50) plus C'. The mean and each value of Ag-V-PFC of two cultures are shown. The same spleen cells as used for virus plaque assay were cultured for 4 days, and the effect of ATS treatment on antibody-producing cells was also tested (C). (□), direct anti-SRBC PFC; (▨), indirect anti-SRBC PFC. Mean value  $\pm$  standard error of three cultures are shown.

without affecting the background level of V-PFC (17). The following experiments were performed to investigate whether or not the Ag-V-PFC generating in the culture were indeed T cells. Spleen cells from mice primed 4 or 5 days previously with  $10^7$  SRBC were cultured with or without SRBC for 2 days. The cultured cells were treated with ATS plus C' or normal rabbit serum (NRS) plus C', and then the virus plaque assay was performed. As shown in Fig. 2 A and B, treatment with ATS (1:50-1:25) eliminated 80-90% of Ag-V-PFC. Background V-PFC (about  $600/10^6$  treated cells) were scarcely affected by the same treatment. Since ATS used here never injured B cells (14) nor anti-SRBC PFC (Fig. 2 C), it was strongly suggested that most of Ag-V-PFC belonged to T-cell population. A lower concentration of ATS (1:100) was not sufficient enough to eliminate Ag-V-PFC.

*Antigen Specificity of Ag-V-PFC.* Spleen cells from mice primed 5 days previously with  $10^7$  SRBC were cultured in the presence of either SRBC or HRBC, or without the addition of antigen. Virus plaque assay was performed on the 2nd day, and antibody response to both SRBC and HRBC were assayed on the 4th day.

A considerable number of Ag-V-PFC was generated in HRBC-stimulated culture, the level of Ag-V-PFC being about 40% of that in SRBC-stimulated culture (Table I). Anti-HRBC antibody response was also observed in the

TABLE I  
*Cross-Reactivity at the Level of Ag-V-PFC for SRBC vs. HRBC\**

In vitro Ag	V-PFC/10 <sup>6</sup> plated cells‡	Ag-V-PFC/10 <sup>6</sup> plated cells§	Percent cross-reaction
None	1,138 ± 186	—	—
SRBC	9,000 ± 1,298	7,862	100
HRBC	4,483 ± 911	3,345	42

\* Spleen cells from mice primed intravenously with 10<sup>7</sup> SRBC 5 days previously were cultured in the presence of 10<sup>7</sup> SRBC or 10<sup>7</sup> HRBC, or in the absence of antigen. 2 days after culture, a V-PFC assay was performed.

‡ The data are expressed as the mean value of V-PFC ± the standard error of the mean for triplicate culture.

§ Ag-V-PFC was calculated as described in Materials and Methods.

TABLE II  
*Antigen Specificity in Anti-SRBC Antibody Response\**

In vitro Ag	Anti-SRBC PFC/culture‡		Anti-HRBC PFC/culture‡	
	Direct	Indirect	Direct	Indirect
None	460 ± 120	380 ± 76	167 ± 47	7 ± 7
SRBC	8,853 ± 350	5,733 ± 228	100 ± 12	13 ± 7
HRBC	360 ± 64	430 ± 72	1,300 ± 220	200 ± 15
Percent cross-reaction	0	0.9		

\* Spleen cells from mice primed intravenously with 10<sup>7</sup> SRBC 5 days previously were cultured in the presence of 10<sup>7</sup> SRBC or 10<sup>7</sup> HRBC, or in the absence of antigen. 4 days after culture, anti-SRBC PFC and anti-HRBC PFC were assayed.

‡ Each value represents the mean of PFC ± the standard error of the mean for triplicate culture.

HRBC-stimulated culture of SRBC-primed spleen cells (Table II). This may probably be attributable to the cross-reaction at the helper T-cell level, since primary anti-HRBC PFC response was not observed in these culture conditions (data not shown), and since no cross-reaction was found at the level of PFC (Table II). Thus, it was suggested that the cross-reactivity of helper T cells for SRBC vs. HRBC was about 10%.

Cross-reactivity of DTH for SRBC vs. HRBC was also investigated. Mice primed with an intravenous injection of 10<sup>7</sup> SRBC 3 days previously, and normal mice as the control, were challenged with 10<sup>8</sup> SRBC into one footpad and 10<sup>8</sup> HRBC into the other footpad. The swelling was measured 24 h later, and the results were shown in Table III. Delayed reaction to HRBC was about 20% of that to SRBC.

These observations suggested that the degree of cross-reactivity in Ag-V-PFC for SRBC vs. HRBC may be comparable to or greater than that in helper T-cell activity or in DTH.

*Effect of Antiserum on the In Vitro Generation of Ag-V-PFC.* It is well known that the development of anti-SRBC antibody-forming cells is suppressed by specific antibody without affecting the generation of helper T cells (20). Recently it was shown by Lagrange et al. (19) that DTH to SRBC was sup-

TABLE III  
*Cross-Reactivity at the Level of DTH for SRBC and HRBC\**

Test Ag	Footpad swelling at 24 h ( $1/10$ mm) $\ddagger$		Net swelling	Percent cross-reaction
	Normal mice	Primed mice		
SRBC	3.0 $\pm$ 0.29	8.0 $\pm$ 1.02	5.0	100
HRBC	2.86 $\pm$ 0.38	4.0 $\pm$ 0.35	1.14	23

\* Mice were immunized intravenously with  $10^7$  SRBC or injected with saline as the control. 3 days later, an eliciting dose ( $1 \times 10^8$ ) of SRBC was injected into one footpad and the same dose of HRBC into the other footpad. Footpad swellings were measured 24 h later and expressed in the unit of  $1/10$  mm.

$\ddagger$  The data are expressed as mean of the swellings  $\pm$  the standard error of the mean for four mice.

pressed by anti-SRBC serum when given either induction or manifestation phase. In the latter experiments, absorbed anti-SRBC serum (absorbed with SRBC) was more effective than native anti-SRBC serum (19). Thus, it seemed to be a hopeful clue for the characterization of Ag-V-PFC to investigate the effect of anti-SRBC serum or the absorbed serum. Spleen cells were taken from mice primed by an intravenous injection of  $10^7$  SRBC 5 days previously and cultured in the absence or in the presence of SRBC or heavily conjugated TNP-SRBC. Anti-SRBC serum (US4) or absorbed antiserum (AS4), prepared as described in Materials and Methods, was added to the culture medium at the onset of culture. Virus plaque assay was performed 2 days after the culture, and anti-TNP and anti-SRBC PFC were assessed on day 4. The results are shown in Fig. 3. High concentration of US4 (2.5%) completely suppressed the generation of Ag-V-PFC, while the same concentration of AS4 had no significant effect (Fig. 3 C). On the other hand, helper T-cell activity was not suppressed by the same dose of US4, but was rather "apparently" enhanced (Fig. 3 B). Anti-SRBC antibody response was suppressed by both US4 and AS4. These results suggested but did not prove that the antigen-specific T cell detected as Ag-V-PFC did not represent the helper T cell itself.

*Effect of Cyclophosphamide (CY) Pretreatment.* Injection of CY before the immunization is known to enhance DTH against SRBC in mice (21), or against protein antigens in guinea pigs (22). If Ag-V-PFC represent effector T cells in DTH, it can be expected that the generation of Ag-V-PFC may be greater in spleen cells from CY-pretreated mice than in those from CY-nontreated mice, since spleen cells from CY-pretreated mice are far more effective in transferring DTH to normal mice (21). CY (200 mg/kg) was injected intravenously into mice 2 days before immunization with  $10^7$  SRBC. Control mice were given an injection of saline instead of CY. 6 days after immunization, spleen cells were harvested and cultured with or without SRBC for virus plaque assay, as well as for the assay of anti-SRBC antibody response. DTH was assayed simultaneously in the separate groups of mice.

DTH on the 6th day after immunization was markedly augmented by the pretreatment with CY (Fig. 4 C), conforming to the data obtained by Lagrange and Mackaness (21). On the other hand, the number of Ag-V-PFC in the culture of spleen cells from CY-pretreated mice was only slight (Fig. 4 A). The results shown in Fig. 4 B indicated that anti-SRBC antibody response was also pro-

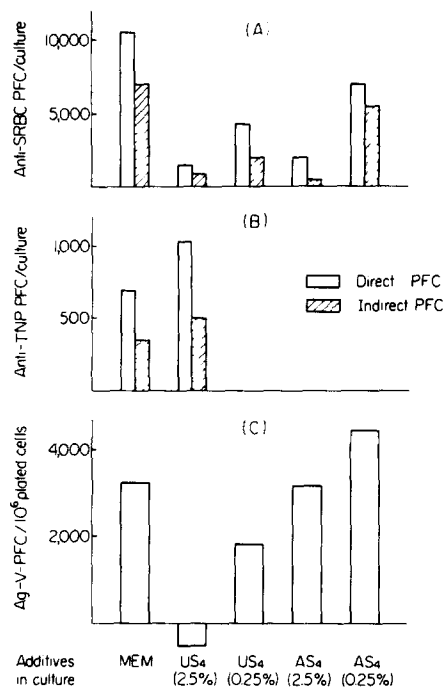


FIG. 3. Effects of anti-SRBC serum (US4) or absorbed anti-SRBC serum with SRBC (AS4) on the generation of anti-SRBC PFC (A), anti-TNP PFC (B), and Ag-V-PFC (C). Spleen cells from mice primed with  $10^7$  SRBC 5 days previously were cultured in the absence or presence of SRBC or heavily conjugated TNP-SRBC. At the onset of culture, US4 (final 2.5 or 0.25%), AS4 (final 2.5 or 0.25%), or medium (MEM) as the control was added in the cultures. Virus plaque assay was performed on the 2nd day of culture, and anti-SRBC and anti-TNP PFC were assessed on the 4th day. Each value represents the mean of three cultures.

hibited by CY pretreatment. These experiments revealed the absolutely contrary effect of CY upon the development of T cells detected as Ag-V-PFC and that of DTH, suggesting the quite different nature of Ag-V-PFC from DTH-mediated effector T cells.

*Kinetic Study of the Development of Ag-V-PFC after Immunization.* The following experiments were performed to investigate the development of T cells detected as Ag-V-PFC after immunization in reference to DTH. Mice were given an intravenous injection of  $10^5$ ,  $10^7$ , or  $10^9$  SRBC and killed various days after immunization. Their spleen cells were cultured for 2 days with or without SRBC, and then virus assay was performed. DTH was also checked in the separate groups of mice. Results are shown in Figs. 5-7. In these figures, levels of Ag-V-PFC are recorded against the day when the spleen cells were harvested, and delayed reactions are recorded against the day when the test antigen was injected into the footpad. In the group of mice primed with  $10^5$  SRBC, the peak level of DTH was attained on the 4th day after immunization, and declined thereafter (Fig. 5). In contrast, Ag-V-PFC seemed to develop after DTH diminished, and attained the peak on the 8th day. The high level of Ag-V-PFC seemed



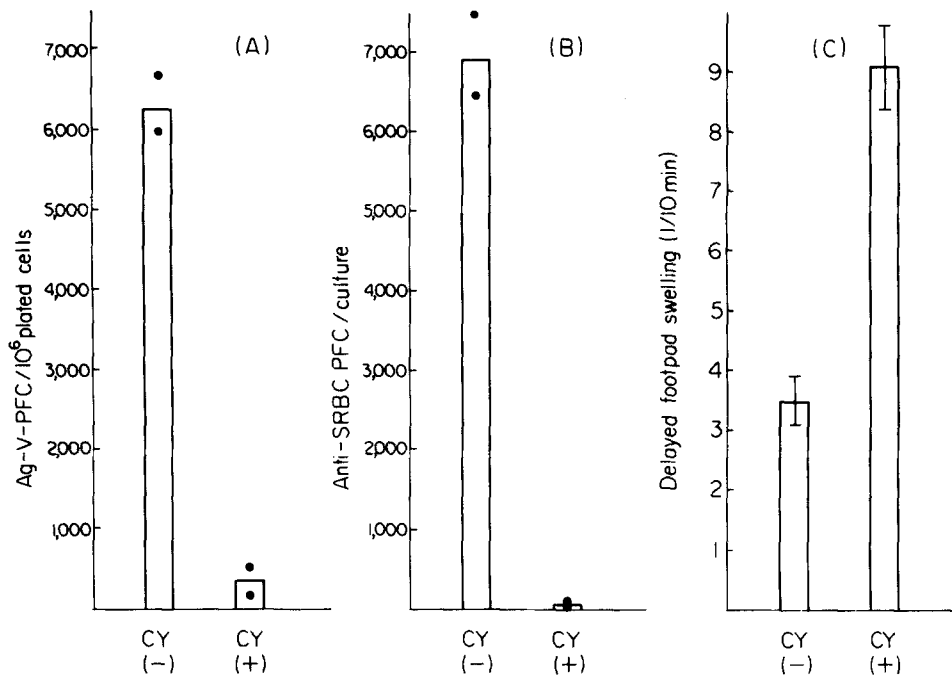


FIG. 4. Abrogation of the development of Ag-V-PFC by a pretreatment with CY. Mice were injected intravenously with CY ( $200 \text{ mg} \cdot \text{kg}^{-1}$ ) or saline and 2 days later immunized intravenously with  $10^7$  SRBC. 6 days after immunization, spleen cells were cultured with or without the addition of SRBC. Ag-V-PFC (A) were assayed on the 2nd day of culture, and anti-SRBC PFC (B) were assayed on the 4th day. DTH (C) was also assayed 6 days after immunization. In (A) and (B), the mean and each value of two cultures are shown. In (C), the mean and the range of standard error are shown.

to be sustained rather a long time after immunization, the number being about 2,000 per  $10^6$  plated cells on the 16th day.

The levels and kinetic patterns of development of both DTH and Ag-V-PFC in mice primed with  $10^7$  SRBC resembled those in mice primed with  $10^5$  SRBC (Fig. 6). Only the time span of the latent phase seemed to be 2-3 days shorter. In mice primed with  $10^8$  SRBC, Ag-V-PFC began to develop as early as the 2nd day and attained the peak on the 4th day after immunization, though DTH was not conspicuously detected in this group (Fig. 7). The level of Ag-V-PFC declined thereafter to become undetectable by day 16.

### Discussion

The development of the virus-replicating lymphocytes was studied in the immune response of mice to SRBC. Cells that acquired the capacity to replicate VSV by antigenic stimulation (Ag-V-PFC) were generated in the culture of spleen cells from mice immunized with SRBC. In the previous paper (17), it was shown that the generation of Ag-V-PFC *in vitro* was completely abolished by the treatment of primed spleen cells with ATS plus C' before culture. Experiments presented here showed that treatment of cultured cells with ATS plus C' eliminated 80-90% of Ag-V-PFC. Thus, it was revealed that most of Ag-V-PFC

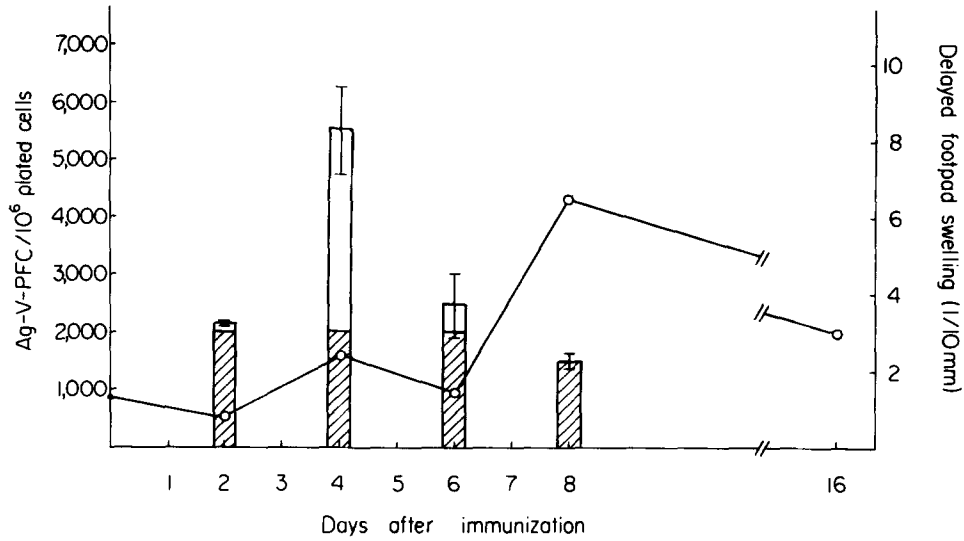


FIG. 5. Kinetics of the development of Ag-V-PFC and DTH after the intravenous injection of  $10^5$  SRBC. Mice were immunized with  $10^5$  SRBC, and various days after immunization spleen cells were cultured with or without SRBC. Virus plaque assay was performed after 2 days of culture. DTH was also assayed in the separate group of mice. The level of Ag-V-PFC (○—○) was recorded against the day when spleen cell culture was set up, and DTH (□) was recorded against the day when eliciting dose of SRBC was injected into the footpad. Hatched area of the column represents the background swelling, i.e., footpad swelling of nonprimed mice by an eliciting dose of SRBC.

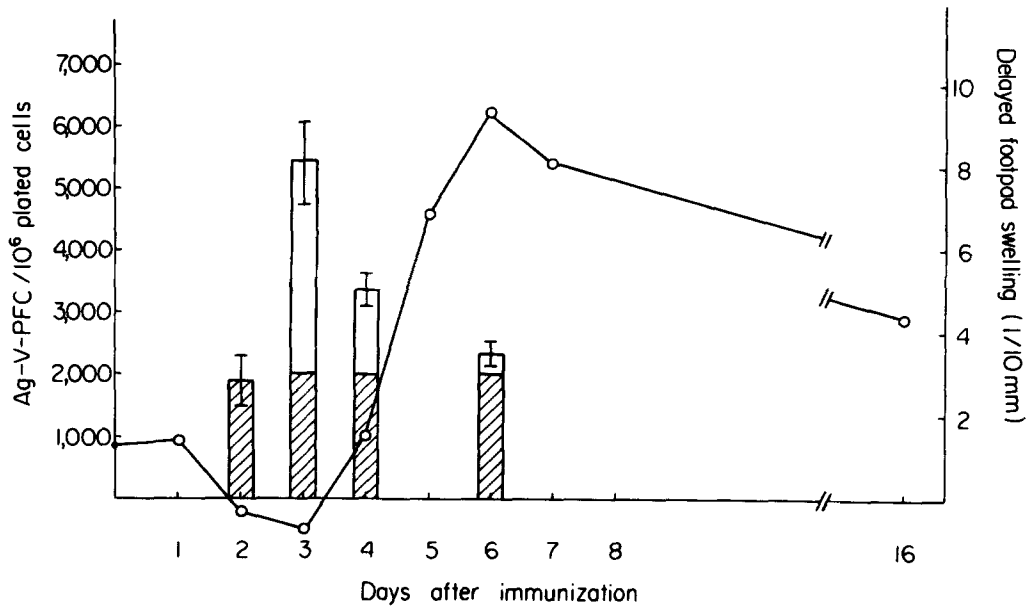


FIG. 6. Kinetics of the development of Ag-V-PFC and DTH after the intravenous injection of  $10^7$  SRBC. Experimental protocol and indications are the same as in Fig. 5.

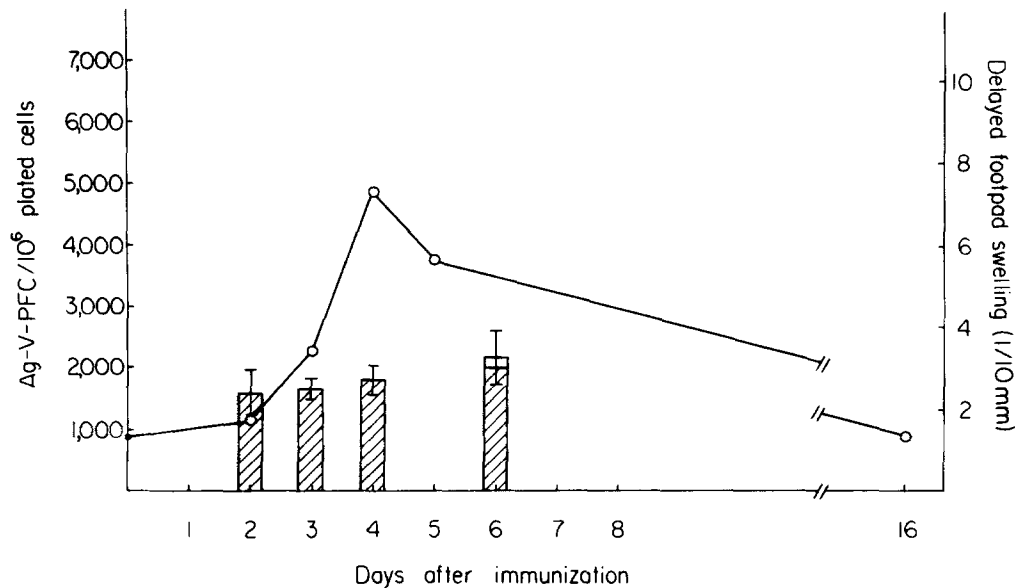


FIG. 7. Kinetics of the development of Ag-V-PFC and DTH after the intravenous injection of  $10^9$  SRBC. Experimental protocol and indications are the same as in Fig. 5.

indeed belonged to T-cell population. It was noted here that two to fourfold higher concentration of ATS was required for the almost complete elimination of Ag-V-PFC in the case of cultured spleen cells compared with the case of spleen cells before culture. This is consistent with the findings that activated T cells were more resistant to immune cytolysis by anti- $\theta$  treatment (8, 23).

"Broad" cross-reaction (about 40%) for SRBC vs. HRBC in the generation of Ag-V-PFC (Table I) confirmed that Ag-V-PFC belonged to T cells, since little cross-reaction was observed at the level of antibody response (Table II). "Broad" cross-reactivity between heterologous erythrocytes at the level of T cells was also observed by other investigators in various assay systems, such as rosette-forming cells (24), killer cells (25), DTH (25), and helper cells (25, 26). In our present experiments, the degree of cross-reactivity of Ag-V-PFC for SRBC vs. HRBC was comparable to or greater than that of DTH (about 20%) or that of helper activity (about 10%).

The kinetic patterns of the generation of Ag-V-PFC in the culture (Fig. 1) were quite different from the pattern observed in the virus plaque assay of lymphocytes from tuberculin hypersensitive donors stimulated with PPD *in vitro* (7), in which linear increase of Ag-V-PFC was seen for at least 4 days during the culture. If we accept that the antigen-reactive lymphocytes detected by Bloom et al. (7) and those detected by us belong to the same cell population, the difference in the pattern of the generation *in vitro* may be attributable to the difference in the experimental systems, such as antigens or animals used. It seemed unlikely that the decline of Ag-V-PFC by the 3rd day in the present study was ascribed to the non-specific cell death during the culture, since the cell viability of the 2nd and the 3rd day did not differ so much, about 40-50% of cells being viable on these days. Thus, the decline of Ag-V-PFC by the 3rd day

may be explained by either or all of the following: (a) The cells responsible for Ag-V-PFC in SRBC-stimulated cultures were exhausted rapidly under the sustained antigenic stimulation *in vitro*. (b) As suggested by Kano et al. (9), replication of VSV occurred maximally in the earlier restricted stage of lymphocyte activation. (c) Specific antibody produced in the culture inactivated the lymphocytes responsible for Ag-V-PFC. We have not investigated whether the first or the second possibility may explain the rather abrupt reduction of Ag-V-PFC during 3 days. However, the third possibility was thought to explain the characteristic pattern observed in our experiments, because anti-SRBC antibody was shown to be produced in the culture (17), and passively administered anti-SRBC antiserum in the cultures almost completely suppressed the generation of Ag-V-PFC (Fig. 3). It is probable that the linear increase of PPD-reactive V-PFC observed by Bloom et al. (7) might have resulted from lack of the production of specific antibody affecting VSV-replicating lymphocytes in the culture. Thus, we may be allowed at present to consider that Ag-V-PFC detected in the present experiments and those by Bloom et al. (7) belong to the same population of cells, until experimental evidence against such an assumption are adduced.

The generation of Ag-V-PFC in the cultures of spleen cells from mice primed 5 days previously with varying doses of SRBC seemed to correlate well with that of helper activity (Fig. 1). It should be noted, however, that anti-hapten antibody response does not necessarily reflect the net helper activity, since the interaction between hapten-specific B cells and helper T cells may be strongly interfered with by the existence of carrier specific B cells (20, our unpublished data). The addition of anti-SRBC antiserum into the culture enhanced the anti-TNP antibody response of SRBC-primed spleen cells. Such enhancement was more prominent in the culture of spleen cells from mice primed with  $10^9$  SRBC (unpublished data). The enhancement of anti-hapten antibody response by anti-carrier antiserum may probably be attributable to the abrogation of competition between hapten-specific B cells and carrier-specific B cells for helper T cells, since the addition of anti-SRBC antiserum in the culture completely suppressed the generation of anti-SRBC PFC *in vitro* (Fig. 3). The net activity of helper T cells may be measured only in such experimental systems in which B cells directed to the carrier are eliminated. Therefore, the experimental data on helper function as shown in Fig. 1 should be reconsidered with reference to the concept of the competition between hapten-reactive and carrier-reactive B cells. Either way, it was shown that helper activity was not suppressed by specific antiserum (Fig. 3B). On the other hand, the generation of Ag-V-PFC was completely suppressed by the same dose of antiserum (Fig. 3C). These results suggested that the antigen-reactive T cell detected as Ag-V-PFC belonged to a different population of cells from helper T cells, though the possibility could not be eliminated that the specific antibody blocked some metabolic stages of helper T cell in which VSV was replicated, without affecting helper function. It was also improbable that Ag-V-PFC represented the precursor of helper T cell. Thus, in mice primed with  $10^5$  SRBC, Ag-V-PFC developed to attain the peak 8 days after immunization (Fig. 5), while helper activity was the highest as early as 4 days (18, our unpublished data).

The suppressive effect of anti-SRBC serum and the absorbed serum on DTH against SRBC was thoroughly studied by Mackaness et al. (13). They showed that the suppressive effect of the absorbed serum was stronger than the native antiserum, and that spleen cells from such suppressed mice were ineffective in transferring DTH to normal mice. In contrast, the absorbed serum had virtually no effect on the *in vitro* generation of Ag-V-PFC (Fig. 3). Though it is difficult to compare the results obtained in *in vitro* experiments with those in *in vivo* experiments, the dissociative effect of the absorbed serum suggested the difference of Ag-V-PFC from effector cells in DTH.

Kinetic studies (Figs. 5-7) showed that the latent period for the development of Ag-V-PFC became shorter as the immunizing doses increased. The peak level of Ag-V-PFC, on the other hand, did not necessarily depend on the immunizing doses. In the group primed with  $10^5$  or  $10^7$  SRBC, Ag-V-PFC developed after the level of DTH diminished. Comparable level of Ag-V-PFC was observed in the group primed with  $10^9$  SRBC, though DTH did not develop in this group. These results strongly suggested that Ag-V-PFC did not represent functional effector cells in DTH nor their precursors.

Concerning the mechanisms of the enhancement of DTH by CY pretreatment, it was suggested that CY pretreatment eliminated the B-cell responses which had suppressive influence upon DTH (21, 22, see also Fig. 4). On the other hand, Askenase et al. (27) showed that augmentation of DTH against SRBC was equally caused by the low dose of CY which had no effect on antibody response. Moreover, the augmentation of DTH by CY pretreatment was also shown in mice immunized with low dose of SRBC by which no antibody response was induced (27). Thus, the existence of suppressive influence upon DTH by the CY-sensitive cells other than antibody-forming cells was strongly suggested. Recent work by Askenase et al. (28) presented the evidence for the existence of suppressor T cells involved in DTH against SRBC. In the present experiments, treatment of mice with CY before immunization completely suppressed the development of Ag-V-PFC and markedly enhanced DTH when both were assayed on the same day (Fig. 4). Thus, the Ag-V-PFC seemed to have similar character to suppressor T cells in DTH. Our unpublished data showed that injection of VSV together with antigen markedly augmented DTH against SRBC or other protein antigens without any noticeable effects on antibody production (manuscript in preparation). This augmentative effect of VSV was antigen specific. Since VSV was shown to be a lytic virus (9), these observations again suggested that Ag-V-PFC may not represent the effector cells, but might represent the cells involved in the regulation of DTH. More detailed studies, of course, seem to be necessary before the exact nature of lymphocytes which permit VSV replication can be clarified. We hope that the clue for the further investigation can be found in the recent study by Cantor and Simpson (29) in which it was shown that the induction of killer T cells was regulated by a distinct subpopulation of T cells.

### Summary

Virus plaque-forming cell assay with vesicular stomatitis virus (VSV), which had been originally introduced by Bloom and his colleagues as a tool for the enumeration of activated lymphocytes, was first applied to the immune response of mice to a widely used antigen, i.e. sheep red blood cells (SRBC). When spleen

cells taken from mice previously primed with SRBC were cultured in the presence of the antigen, lymphocytes capable of replicating VSV (antigen-induced virus plaque-forming cells, Ag-V-PFC) were generated in the culture. They seemed to appear as early as 1 day of culture, and the peak was attained by the 2nd day. Most of Ag-V-PFC belonged to T-cell population, since 80-90% of Ag-V-PFC was killed by the treatment of cultured cells with anti-thymocyte serum plus complement.

*In vitro* generation of Ag-V-PFC seemed to be highly cross-reactive (about 40%) with a related antigen (horse red blood cells). Ag-V-PFC detected in the present experiment may not represent helper T cells, effector T cells, or their precursors because of the following: (a) The generation of Ag-V-PFC was completely suppressed by the addition of anti-SRBC mouse serum in the culture, though the helper activity was apparently augmented by the same treatment. (b) Development of Ag-V-PFC was almost completely suppressed by the pre-treatment of mice with cyclophosphamide 2 days before immunization, by which delayed-type hypersensitivity (DTH) was markedly augmented. (c) After the immunization of mice, Ag-V-PFC began to develop just when the level of DTH declined, at which point helper activity of the spleen cells also diminished. A possible role of Ag-V-PFC in the immune response was discussed.

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