VIRUS-REPLICATING T CELLS IN THE IMMUNE RESPONSE OF MICE
II. Characterization of T Cells Capable of Replicating Vesicular Stomatitis Virus*

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Virus plaque assay (VPA), an assay system for T-cell activity, originally developed by Bloom et al. (1), is a method with a unique property by which the number of activated T cells can be directly counted. VPA is based on the observation that activated T cells are especially vulnerable to the infection of some kinds of viruses, including vesicular stomatitis virus (VSV). Recently, many authors gave evidence that the T-cell population comprises heterogeneous subpopulations with respect to their distribution and circulating patterns (2, 3), life span (4, 5), cell membrane antigenicity (6), response to mitogens (7), presence of Fc receptor (8), or adherence to nylon wool columns (9). It is necessary to determine whether all the T cells are susceptible to virus infection, or only a restricted T-cell subpopulation permits virus replication after the stimulation by antigens.

In the previous paper (10), we reported that antigen-reactive, VSV-replicating T cells were generated in the antigen-stimulated culture of spleen cells from mice immunized with sheep erythrocytes (SRBC). Development of VSV-replicating cells occurred after the peak of delayed-type hypersensitivity (DTH), and was suppressed by the treatment of mice with cyclophosphamide resulting in the enhancement of DTH. Thus we suggested that VSV-replicating T cells are not the effector T cells in DTH, but may be the T cells involved in the regulation of DTH.

The present study was performed to investigate in detail the characteristics of VSV-replicating T cells with special reference to T cells involved in the humoral immune response. Results indicate that helper T cells do not develop to VSV-replicating cells. VSV-replicating T cells are shown to be antigen reactive and nylon wool-adherent T cells, which are derived from the Tc population.

Materials and Methods

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

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Abbreviations used in this paper: Ag-V-PFC, antigen induced virus plaque-forming cells; ALS, anti-mouse lymphocyte serum; Ara C, cytosine arabinoside; ATx, adult thymectomy; DTH, delayed-type hypersensitivity; DNP-KLH, dinitrophenylated keyhole limpet hemocyanin; FCS, fetal calf serum; HRBC, horse erythrocytes; 2-ME, 2-mercaptoethanol; MEM, Eagle's minimal essential medium; MMC, mitomycin C; Mo, macrophages; PEC, peritoneal exudate cells; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TNP, trinitrophenol; Tv, the T cell which acquires the capacity of VSV-replication by the antigenic challenge in vitro; VPA, virus plaque assay; VSV, vesicular stomatitis virus.
Adult Thymectomy (ATx). Thymectomy of young adult mice (4- to 5-wk old) was performed as previously described (11).

Conjugation of 2,4,6-Trinitrophenol (TNP) to Erythrocytes. Heavily conjugated TNP-SRBC was prepared according to Kettman and Dutton (12) for the in vitro assay of the carrier effect. The preparation of TNP-conjugated horse erythrocytes (TNP-HRBC) for the assay of anti-TNP antibody-forming cell was essentially the same as that reported by Rittenberg and Pratt (13).

Cell Separation by Nylon Wool Column. Spleen cells were fractionated by a nylon wool column according to the method of Julius et al. (14) with slight modification. Nylon wool (Fenwal Laboratories, Morton Grove, Ill.) was boiled three times in twice distilled water, and 1 g in dry weight was packed into the 20-ml syringe to be sterilized in an autoclave. Before the application of cells, nylon wool was rinsed and saturated with Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (FCS). 1 × 10^8 Spleen cells in 5 ml of FCS-MEM, were charged to the column and incubated at 37°C for 1 h in humidified air-7% CO2. After incubation, nonadherent cells were eluted with 20 ml of effluent medium. Nylon wool adherent cells were then pushed out by compressing the nylon wool with the plunger. This was reported three times by adding 5 ml of FCS-MEM each. Viable cell recovery was ≈20% in nonadherent fractions and ≈55% in adherent fractions.

Deprivation of Macrophages. Macrophages were deprived from spleen cells according to the method of Ly and Mishell (15) by using a Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden) column. Briefly, 10 g of glass beads were put on the nylon fiber layer at the bottom of a 50-ml syringe, 10 ml of 5% FCS-MEM was added, and 40 ml of Sephadex-saline slurry was poured gently onto the beads. After washing with 150 ml of warm FCS-MEM, 4 ml of spleen cell suspension, 1.5 × 10^8/ml, was loaded on the Sephadex, and eluted with 20 ml of FCS-MEM. Final recovery of viable cells was about 40%.

Preparation of Peritoneal Exudate Cells (PEC). PEC were prepared from mice given intraperitoneal injection of 1 ml thioglycollate medium (Nissui Seiyaku, Tokyo, Japan) 3 days previously. Cells were washed twice with MEM at 4°C, and resuspended in 8% FCS-MEM.

Irradiation of Spleen Cells. Spleen cell suspension in 8% FCS-MEM at a concentration of 1 × 10^7/ml was X-irradiated with a soft X-ray emitter (Softex CMBW, Softex Co., Ltd., Tokyo, Japan) which delivers 500 rads per min. Spleen cells received 2,500 rads in 5 min.

Assay for Helper T-Cell Activity. Helper activity of the SRBC-primed spleen cells was assayed in vitro in the anti-TNP antibody response. Normal spleen cells or spleen cells from mice injected intraperitoneally with 1 ml dinitrophenylated keyhole limpet hemocyanin (DNP-KLH) in Freund's complete adjuvant 4- to 8-wk previously were used as a B-cell source. Concentration of spleen cells was adjusted to 1 × 10^7 cells/ml in 8% FCS-MEM containing 5 × 10^-5 M 2-mercaptoproethanol (2-ME). 0.1 ml of either normal or DNP-KLH-primed spleen cells (4 × 10^6) in the presence of 5 × 10^6 TNP-SRBC in Linbro plastic multidishes (Linbro Chemical Co., New Haven, Conn.) for 4 days. Viable cell recovery was 20% in nonadherent fractions and 35% in adherent fractions.

VPA. VPA was performed as described previously (10). Briefly, spleen cells from mice immunized with SRBC were cultured with or without SRBC (1 × 10^7) at a concentration of 1 × 10^7/ml in 1.5 ml of culture medium in 35-mm plastic dishes (Falcon Plastics, Oxnard, Calif.). 2-ME was added at 5 × 10^-5 M in the culture medium unless otherwise stated. After 2 days of culture, cells were harvested, washed by centrifugation, and portions of 5 × 10^6 cells were resuspended in 0.2 ml of MEM containing 1 × 10^7 plaque-forming units of VSV. The cell suspension was incubated at 37°C for 2 h in a humidified air-7% CO2 incubator for virus adsorption, and then washed three times. Afterwards they were incubated with anti-VSV serum at 4°C for 1 h to inactivate free viruses. After two washings, 1 × 10^6 of the infected cells in 0.1 ml were added on the medium-drained monolayers of L cells, and 1% agar in 6% FCS-MEM was poured and gently mixed with cells. Each sample group was tested in triplicate. The dishes were incubated at 37°C in a humidified air-7% CO2 incubator for 1-2 days, and stained with neutral red. Antigen induced V-PFC (Ag-V-PFC) were calculated by subtracting the mean number of virus plaques in the control cultures from those in the antigen-stimulated cultures.
Table I

**Effect of Mitotic Inhibitors on the Generation of Ag-V-PFC and Helper T-Cell Activity**

| Exp. | In vitro antigen | Inhibitor | Ag-V-PFC*/10⁶ Plated cells | [³H]Thymidine uptake (cpm/culture)‡ | Indirect anti-TNP PFC/culture‡ |
|------|-----------------|-----------|-----------------------------|--------------------------------|--------------------------------|
| I    | −               | −         | −                           | 5,420 ± 370                   |                                 |
|      | +               | −         | 3,060                       | 9,310 ± 260                   |                                 |
|      | +               | Vinblastine§ | 530                        | 920 ± 100                     |                                 |
| II   | −               | −         | −                           | 43 ± 21                       |                                 |
|      | +               | −         | 3,500                       | 330 ± 180                     |                                 |
|      | +               | MMC¶      | −100                        | 437 ± 140                     |                                 |
|      | +               | Anti-SRBC¶ | 600                         | 1,130 ± 350                   |                                 |

Spleen cells from mice immunized with 10⁷ SRBC 6 days previously were cultured with or without SRBC. VPA was performed on the 2nd-day, and [³H]thymidine incorporation was examined on the 3rd-day. For the assay of helper activity, the same spleen cells as above were filtered through a nylon wool column, and 1 × 10⁶ effluent cells were cultured together with 4 × 10⁶ DNP-KLH primed spleen cells in the presence or absence of TNP-SRBC. Indirect anti-TNP PFC were determined on the 4th-day.

* Calculated by subtracting the mean number of V-PFC in antigen free cultures from those in the antigen-stimulated cultures.

‡ Mean ± SE for triplicate cultures.

§ 1 µg/ml vinblastine was added into the culture at the onset.

¶ Spleen cells or nylon wool column passed spleen cells were incubated with 20 µg/ml MMC at 37°C for 30 min, washed, and cultured for VPA or helper assay.

‖ Anti-SRBC antiserum obtained from mice injected with 10⁹ SRBC i.v. 4 days previously was added into the culture (final concentration, 2.5%).

**Miscellaneous.** Mitomycin C (MMC) (Kyowa Hakko, Tokyo, Japan) and vinblastine sulfate (Eli Lilly and Co., Indianapolis, Ind.) were used as the mitotic inhibitors. To assess DNA synthesis, [³H]thymidine (1 µCi; The Radiochemical Centre, Amersham, U.K.) was added to the culture for a 16-h period. Rabbit anti-mouse lymphocyte serum (ALS) was purchased from Microbiological Associates (Walkersville, Md.). Rabbit antiserum specific to mouse T cells was produced as previously described (17).

**Results**

**Effect of Mitotic Inhibitors on the Generation of Ag-V-PFC in Vitro.** Spleen cells from mice primed with 10⁷ SRBC 6 days previously were cultured with or without SRBC. 1 µg/ml Vinblastine was contained in one culture group throughout the culture period. VPA was carried out on the 2nd-day, and DNA synthesis was examined on the 3rd-day by pulsing with [³H]thymidine for 16 h. As shown in Table I (Exp. I), the generation of Ag-V-PFC as well as DNA synthesis was almost completely inhibited by vinblastine.

The effect of MMC, another mitotic inhibitor, on the generation of Ag-V-PFC was examined in comparison with the effect on the helper T-cell activity. SRBC-primed spleen cells were treated with MMC (20 µg/ml) for 30 min at 37°C, washed, and then cultured with SRBC for VPA. For assaying helper T-cell activity, nylon wool-passed spleen cells were treated with MMC as mentioned above, washed, and cultured together with DNP-primed spleen cells and TNP-SRBC. Effect of anti-SRBC antiserum (final 2.5%) on the generation of both Ag-V-PFC and helper T-cell activity was also examined. As shown in Table I (Exp. II) the generation of Ag-V-PFC was completely abolished by the pretreatment with MMC, whereas helper T-cell activity was slightly augmented. Similarly, generation of Ag-VPFC was almost completely
abrogated by the addition of anti-SRBC antiserum to the culture, whereas helper T-cell activity was markedly augmented. This conforms to the previous results (10). These results indicate that Ag-V-PFC are the T cells that entered the mitotic cycle after the antigenic stimulation in vitro.

### Requirement of Macrophages (Mφ) in the Generation of Ag-V-PFC.

Spleen cells from mice primed with $10^7$ SRBC 6 days previously were filtered through Sephadex G-10 column to deprive of Mφ. Column-passed cells were mixed with varying numbers of PEC, and cultured with or without SRBC. VPA was performed on the 2nd-day of culture, and anti-SRBC PFC were assayed on the 4th-day. As shown in Table II (Exp. I), the number of Ag-V-PFC developing in G-10 passed cells was about 20% of control. By the addition of PEC at 0.1 or 1% of lymphoid cells, the generation of Ag-V-PFC in vitro was partially restored to 51 or 60% of the control, respectively. PEC in excess (10%), however, seemed to suppress the generation of Ag-V-PFC. Appreciable number of Ag-V-PFC (about 20% of the control) generated without the addition of PEC in G-10 passed cells may be attributed to the presence of 2-ME in the culture. Without 2-ME, generation of Ag-V-PFC was not observed in the culture of G-10 passed cells (Exp. II), and again the generation of Ag-V-PFC was restored by the addition of PEC (Exp. III). Thus, 2-ME seems unable to replace fully the role of PEC, but is effective in promoting the generation of Av-V-PFC.

Ly and Mishell (15) showed that G-10 passed cells were incapable of generating anti-SRBC antibody response in vitro alone, but the capacity was restored by the addition of PEC (Exp. III). However, dependency on Mφ in antibody response in vitro differed from dependency in the generation of Ag-V-PFC. Thus, 0.1% of PEC, which was nearly optimal for the generation of Ag-V-PFC, was insufficient for restoring the anti-SRBC PFC response. Moreover, it was shown that 2-ME was able to completely replace the role of PEC in the anti-SRBC PFC response (Exp. II). These results suggest that the role of Mφ in the generation of Ag-V-PFC in vitro involves a
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TABLE III

Nylon Wool Column Adherence of Tv

| Exp. | Sephadex G-10 column | Nylon wool column | PEC (2 × 10⁶) | V-PFC/10⁶ Plated cells* | Ag-V-PFC/10⁶ plated cells |
|------|----------------------|-------------------|--------------|-----------------|-----------------|
| I    | Effluent Unfractionated | -                | 1,970 ± 200  | 1,900 ± 350     | 70              |
|      |                     +                | 6,330 ± 840      | 1,370 ± 180  | 4,960           |
|      | Nonadherent          +                | 1,070 ± 90       | 670 ± 30     | 400             |
|      | Adherent             +                | 10,370 ± 780     | 5,400 ± 1,130| 4,970           |
| II   | Effluent Nonadherent  +                | 700 ± 70         | 575 ± 190   | 125             |
|      | Adherent             +                | 5,000 ± 290      | 1,425 ± 340  | 3,375           |

Spleen cells from mice primed with 10⁷ SRBC 6 days previously were filtered through Sephadex G-10 column. Effluent cells were then fractionated into nylon wool nonadherent and adherent fractions. Cells of each fraction and unfractionated spleen cells were cultured with or without SRBC in the absence or presence of PEC. VPA was performed on the 2nd-day of culture.

* Mean ± SE for triplicate plating.

Different profile from the role in the secondary antibody response against SRBC.

Nylon Wool Adherence of the T Cell (Tv) Which Acquire the Capacity of VSV-Replication by the Antigenic Challenge in Vitro. This experiment was performed to compare the adherence to nylon wool column of Tv with that of helper T cells. In the experiment of V-PFC, primed spleen cells were first deprived of Mφ by Sephadex G-10 column, to standardize the Mφ number in nylon wool-separated fractions. G-10 passed cells were fractionated into nylon wool nonadherent and adherent fractions, and each fraction (10⁷ cells) was cultured with the constant number (2 × 10⁴) of PEC in the presence or absence of SRBC. VPA was carried out on the 2nd-day of culture. Results of two independent experiments are shown in Table III. The generation of Ag-V-PFC was prominent only in the culture of nylon wood-adherent fractions, and was negligible in the culture of nonadherent fractions.

Helper T-cell activity in nylon wood-adherent and nonadherent cells was also compared. Primed spleen cells were fractionated into nonadherent and adherent fractions, and 1 × 10⁶ cells of each fraction were cultured together with 4 × 10⁶ normal or DNP-primed spleen cells in the presence of TNP-SRBC. Anti-TNP PFC response was assayed on the 4th-day of culture. Results (Table IV) indicated that helper activity was always greater in the culture of nonadherent fraction than in the culture of adherent fraction, regardless of the B-cell source used.

Effect of ATx. Mice were immunized with 10⁷ SRBC at different intervals (2, 5, and 9 wk) after ATx. 6 days after immunization, spleen cells were cultured with or without SRBC for VPA. As a control, age-matched, nonthymectomized mice were equally immunized, and their spleen cells were cultured in the same way. Helper activity in the same spleen cells as used for VPA was also examined after filtration through a nylon wool column. As shown in Table V, Tv began to decline after ATx, and became almost undetectable by the 9th-week. The half life was estimated to be about 5 wk.

Helper activity in the nylon nonadherent fraction of the spleen cells was assayed as anti-TNP antibody response. The level of helper activity of ATx mice was expressed in terms of percent of response of spleen cells taken from nonthymectomized mice (Fig. 1). For the sake of comparison, the pattern of development of Tv after ATx shown in Table V was also plotted with a broken line. Results indicate that helper T
 Helper T-Cell Activity in Nylon Wool Nonadherent and Adherent Cells

| Helper source* (1 × 10⁶) | B-Cell source† (4 × 10⁶) | Direct anti-TNP PFC/culture‡ | Indirect anti-TNP PFC/culture§ |
|--------------------------|--------------------------|-----------------------------|-------------------------------|
| None                     | Normal                   | 17 ± 9                      | 20 ± 6                        |
| Unfractionated           | Normal                   | 143 ± 29                    | ND‡                          |
| NW non-Ad                | Normal                   | 283 ± 33                    | 227 ± 44                      |
| NW Ad                    | Normal                   | 123 ± 9                     | 193 ± 37                      |
| None                     | DNP-Primed               | 80 ± 25                     |                               |
| NW non-Ad                | DNP-Primed               | 407 ± 72                    |                               |
| NW Ad                    | DNP-Primed               | 193 ± 13                    |                               |

* Spleen cells from mice primed with 10⁷ SRBC 6 days after previously were used with or without fractionation by nylon wool (NW) column. Non-Ad, nonadherent; Ad, adherent.
† Normal spleen cells or spleen cells from DNP-KLH primed mice (DNP-primed) were used as a B-cell source.
‡ Mean ± SE for triplicate culture.
§ Not determined.

Effect of ATx on the Development of Tν

| Mice                  | Immunization | Ag-V-PFC/10⁶ plated cells* | Response‡ |
|-----------------------|--------------|-----------------------------|-----------|
|                       |              | Exp. I Exp. II Exp. III Exp. IV | %         |
| Normal                | None         | 770 — — — —               | —         |
| Normal                | 10⁷ SRBC     | 5,800 5,070 6,700 8,700 100 | 100       |
| ATx (2 wk)§           | 10⁷ SRBC     | — 5,220 — — 103            | 103       |
| ATx (5 wk)§           | 10⁷ SRBC     | — — 3,800 — 57             | 57        |
| ATx (9 wk)§           | 10⁷ SRBC     | — — — 815 9               | 9         |

Normal or adult thymectomized mice were injected with saline or 10⁷ SRBC, and 6 days later the spleen cells were cultured for VPA.

* Calculated from the mean V-PFC for each triplicate plating of antigen-stimulated and control cultures.
‡ The responses of the ATx mice were expressed in terms of the percent of that of the nonthymectomized mice.
§ Adult thymectomized mice. The intervals between ATx and immunization are shown in parentheses.

cells in the nylon nonadherent fraction belong primarily to the relatively long-lived T-cell population, the half-life being more than 9 wk. Thus, it was strongly suggested that Tν and helper T cells developed from different T-cell populations.

Effect of ALS in Vivo on the Development of Tν and Helper T-Cell Activity. It is now well accepted that ALS selectively eliminates the recirculating T cells (18). Mice were injected intraperitoneally with 0.09 ml of saline or ALS 2 days before, on the same day, and 4 or 11 days after the immunization with 10⁷ SRBC. Spleen cells were harvested 13 days after the immunization, cultured for VPA, and for the assay of helper activity. In Fig. 2, levels of Tν and helper activity in spleen cells of ALS injected mice were shown as a percent response to those of the control mice injected with saline instead of ALS. The development of Tν was augmented by ALS. The augmentation was most pronounced when ALS was administered 2 days before the harvest of spleens. By contrast, helper activity was more or less reduced by ALS, though the reduction was not so evident in the group given ALS 2 days before immunization.
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Flo. 1. Effect of adult thymectomy on the development of helper T-cell activity and Tv. Normal mice or mice tx 2, 5, or 9 wk previously were immunized with 10^7 SRBC. 6 days later, the spleen cells were harvested and cultured for the assay of helper activity and VPA. For the assay of helper activity, cells were filtered through a nylon wool column, and 1 x 10^6 nonadherent cells were cultured together with 4 x 10^6 normal spleen cells and 5 x 10^6 TNP-SRBC. Direct anti-TNP PFC was assessed on the 4th-day of culture. Responses of ATx mice were expressed as the percent responses of the control, non ATx mice. Data for Tv (Ag-V-PFC) are the same as in Table V. (O--O), anti-TNP-PFC; (O--O), Ag-V-PFC.

Fig. 2. Effect of ALS administered in vivo on the development of helper T-cell activity and Tv. Mice were injected intraperitoneally with 0.09 ml of saline or ALS 2 days before, on the same day, 4 and 11 days after, immunization with 10^7 SRBC. Spleens were harvested 13 days after immunization and the cells were cultured for the assay of helper activity and VPA. Procedures of assay are the same as in Fig. 1. Responses of ALS-infected mice were expressed as the percent responses of the control, saline-injected mice. (O), Ag-V-PFC; (O), anti-TNP-PFC.

Kinetic Study of the Development after Immunization of Tv, Helper T Cells, and Suppressor T Cells. Spleen cells were harvested from mice immunized with 10^7 SRBC 3 or 7 days previously (day 3 spleen cells and day 7 spleen cells, respectively), and helper activity for normal spleen cells or DNP-primed spleen cells was examined by challenging with TNP-SRBC in vitro. Anti-TNP PFC response was assayed on the 4th-day of culture, and shown in Table VI. Helper activity was observed in day 7 spleen cells regardless of the B-cell source. Day 3 spleen cells, on the other hand, expressed significant helper activity only for normal spleen cells (Exp. I). However, strong helper activity for DNP-primed spleen cells was revealed by irradiating the whole or nylon passed day 3 spleen cells before culture (Exp. II). These results suggested that nylon nonadherent, radiosensitive suppressor cells might be involved in day 3 spleen cells, and that they
## TABLE VI

Enhancement of Helper Activity of Day 3 Spleen Cells by X-Irradiation

| Exp. | Helper source (1 × 10^6)* | Treatment | B-Cell source (4 × 10^6)† | Anti-TNP PFC/culture |
|------|---------------------------|-----------|---------------------------|----------------------|
|      |                           |           |                           | Direct               |
|      |                           |           |                           | Indirect             |
| I    | Day 3 spleen              | NW Passed§| Normal                    | 120 ± 13             |
|      |                           |           | DNP-Primed                | 67 ± 13              |
|      | Day 7 spleen              | NW Passed | Normal                    | 10 ± 0               |
|      |                           |           | DNP-Primed                | 20 ± 0               |
|      | None                      | Untreated | DNP-Primed                | 330 ± 85             |
|      |                           |           | DNP-Primed                | 407 ± 63             |
|      | Day 3 spleen              | NW Passed | DNP-Primed                | 420 ± 45             |
|      |                           |           | DNP-Primed                | 417 ± 46             |
|      | Day 7 spleen              | NW Passed | DNP-Primed                | 50 ± 20              |
|      |                           |           | DNP-Primed                | 60 ± 12              |
|      | None                      | Untreated | DNP-Primed                | 303 ± 58             |
|      |                           |           | DNP-Primed                | 313 ± 49             |
|      | Day 3 spleen              | NW Passed | DNP-Primed                | 37 ± 6               |
|      |                           |           | DNP-Primed                | 13 ± 3               |
|      | Day 7 spleen              | NW Passed | DNP-Primed                | 323 ± 12             |
|      |                           |           | DNP-Primed                | 253 ± 44             |
|      | None                      | Untreated | DNP-Primed                | 236 ± 58             |
|      |                           |           | DNP-Primed                | 160 ± 36             |
|      | Day 3 spleen              | NW Passed | DNP-Primed                | 203 ± 3              |
|      |                           |           | DNP-Primed                | 110 ± 15             |
|      | Day 7 spleen              | NW Passed | DNP-Primed                | 190 ± 12             |
|      |                           |           | DNP-Primed                | 187 ± 18             |
|      | None                      | Untreated | DNP-Primed                | 233 ± 32             |
|      |                           |           | DNP-Primed                | 173 ± 20             |

Cells were cultured in the presence of 5 × 10^6 TNP-SRBC, and PFC were assayed on the 4th-day. Mean ± SE for triplicate culture.

* Spleen cells from mice immunized with 10^7 SRBC 3 days previously (day 3 spleen) or 7 days previously (day 7 spleen) were used as a helper source.

† Normal spleen cells or spleen cells from DNP-KLH primed mice (DNP-primed) were used as a B-cell source.

§ Nylon wool column passed.

∥ Cell suspensions received 2,500 rads X-ray irradiation.

### TABLE VII

Suppressor T Cells Effective Only for Hapten-Primed B Cells

| Helper source | DNP-primed spleen cells as a B-cell source (Indirect PFC) | Percent expected | Normal spleen cells as a B-cell source (Direct PFC) | Percent expected |
|---------------|---------------------------------------------------------|------------------|--------------------------------------------------|------------------|
| None          | 50 ± 26                                                 | 27 ± 9*          | 253 ± 17                                        | 48 ± 12          |
| Unirradiated  | 173 ± 18                                                | 187 ± 59         | 433 ± 44                                        | 487 ± 54         |
| Irradiated‡   | 437 ± 90                                                | 433 ± 44         | 98                                              | ND               |
| Unirradiated  | 293 ± 12                                                | 98               |
| plus irradiated |                                                  |                 |
| Unirradiated (ATS + C')§ | 487 ± 54                                              | ND               |

Spleen cells from mice immunized with 10^7 SRBC 3 days previously were used as a helper source. 1 × 10^6 of untreated or irradiated cells, or the mixture of both, were cultured together with 4 × 10^6 of normal spleen cells or spleen cells from DNP-KLH primed mice (DNP-primed). TNP-SRBC was used as the in vitro antigen. Anti-TNP PFC were assayed on the 4th-day of culture.

* Mean PFC ± SE for triplicate culture.

‡ Spleen cell suspensions received 2,500 rads X-ray irradiation before culture.

§ Unirradiated spleen cells were treated with ATS plus guinea pig serum (C') before culture.

expressed their function only when hapten-primed spleen cells were used as a B-cell source.

The presence of suppressor cells in day 3 spleen cells was substantiated in the experiment shown in Table VII. Nonirradiated or 2,500 rads irradiated day 3 spleen...
TABLE VIII
Failure of Day 3 Spleen Cells to Suppress the Generation of Ag-V-PFC in the Culture of Day 7 Spleen Cells

| Cultured cells* | Ag-V-PFC/10⁶§ | Anti-TNP PFC/culture§ |
|-----------------|---------------|-----------------------|
| Day 3 spleen    | 830           | 173 ± 28              |
| Day 7 spleen    | 3,580         | 680 ± 74              |
| Day 3 spleen plus day 7 spleen | 3,470         | 197 ± 55              |

*For the virus plaque assay, 1 × 10⁷ day 3 spleen cells or 1 × 10⁷ day 7 spleen cells, or the mixture of 5 × 10⁶ day 3 spleen cells and 5 × 10⁶ day 7 spleen cells, were cultured with or without SRBC. VPA was performed on the 2nd day of culture. For helper assay, 1 × 10⁶ day 3 spleen cells or 1 × 10⁷ day 7 spleen cells, or the mixture of 5 × 10⁶ day 3 spleen cells and 5 × 10⁶ day 7 spleen cells, were cultured together with 4 × 10⁶ DNP-KLH primed spleen cells and 5 × 10⁶ TNP-SRBC. Indirect anti-TNP PFC were determined on the 4th-day.

§ Calculated from the mean V-PFC for each triplicate plating of antigen-stimulated and control cultures.

§ Mean PFC ± SE for triplicate culture.

Discussion

T cells which were activated by immunization to acquire the capacity of VSV-replication after in vitro antigenic stimulation were tentatively termed as Tv in this paper, and the immunocytological properties of Tv were investigated particularly in the humoral immune response.

In the previous paper (10), it was shown that the development of Tv was completely prohibited by the administration of cyclophosphamide 2 days before the immunization of mice with SRBC. In the present study, in vitro generation of Ag-V-PFC was abolished by the treatment of cells with mitotic inhibitors, whereas helper activity was somewhat augmented by the same treatment (Table I). These results indicate that in anti-SRBC response Ag-V-PFC represent antigen-reactive proliferative T cells. In the secondary mixed lymphocyte culture, however, Senik and Bloom (19) reported that the generation of V-PFC was not affected by the DNA synthesis inhibitor.
VESICULAR STOMATITIS VIRUS REPLICATING T CELLS (cytosine arabinoside, Ara C), though the sensitivity of V-PFC to Ara C progressively increased in relation to the time interval between primary immunization and in vitro challenge. Thus, it seems unlikely that the proliferative capacity is a sole factor to decide whether or not VSV can replicate in the activated T cells.

The results that the function of Mφ in the generation of V-PFC in vitro cannot be replaced by 2-ME suggest the difference between the mechanism of T-cell activation toward V-PFC and that of activation of helper T cells. As shown in Table II and also by Chen and Hirsh (20), function of Mφ in the secondary anti-SRBC antibody response can be completely replaced by 2-ME. These results indicate that helper T cells in anti-SRBC response can be activated in the absence of Mφ. However, 2-ME cannot be substituted for Mφ in the generation of Ag-V-PFC. This seems to suggest that the development of T cells to V-PFC is more strictly dependent on Mφ than that to helper cells.

The most notable finding for Tv in this study may be that the precursor of Ag-V-PFC was nylon wool adherent (Table III). This was in contrast with the result that the major part of the helper T cells was found in nylon wool nonadherent fraction (Table IV). Some degree of helper activity was also found in the adherent fraction (Table IV). However, helper cells in this fraction seemed less mature than those in nonadherent fraction, since they were more sensitive to irradiation and ATx (our unpublished data). Thus it seems that mature helper T cells pass through the nylon wool column, and then they are distinct from T cells which become V-PFC. Recently, special immunological activities of nylon wool adherent T cells have been reported by several authors. They include initiator T cells involved in the induction of GVH (9), culture-induced suppressor T cells in MLR and cytotoxic response (21), and antigen-induced nonspecific suppressor T cells in the proliferative T-cell response (22). Our data to be presented in the next paper (23) will show that nylon adherent fraction includes a kind of suppressor T cell in the antibody response. Thus, nylon adherent T cells so far reported seem to be functionally associated with the regulation of both humoral and cell-mediated immune responses.

One of the most basic and simple expressions of the T-cell differentiation process may be “T1-T2” concept presented by Raff and Cantor (4). T1 cells are defined as short-lived, nonrecirculating T cells, and T2 cells refer to long-lived, recirculating T cells (4, 24). In the present study, it was strongly suggested that Tv belonged to the T1 population, since the development was ATx-sensitive (half life ~5 wk) and ALS-resistant. On the other hand, helper T cells were shown to be derived from both T1 and T2 population, conforming to the finding by Kappler et al. (5). Thus, in our experiments, helper T cells detected in the nylon adherent fraction seem to be derived from the T1 population, since the activity tended to decline rather rapidly after ATx (unpublished data). Helper T cells in the nylon nonadherent fraction, on the other hand, seem to be derived from the T2 population, since they were relatively long-lived (half life being more than 9 wk) and became increasingly ALS sensitive after immunization. The relative ALS resistance of nylon nonadherent helper T cells in the early phase after immunization might be explained by the selective sequestration of these T cells in the spleen as described by Sprent and Miller (25). Their experiments showed that recirculating lymphocytes were sequestered for 1 or 2 days into the organs where the antigen was retained, and then abruptly re-entered the recirculating pool.

The results concerning the kinetics of helper T-cell development were somewhat
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TABLE IX
Characteristics of Tv

| Antigen reactive T cell. |
|--------------------------|
| The antigen specificity is fairly "broad", compared with B cell. |
| In vitro generation is dependent on the presence of macrophages. |
| In vitro generation is suppressed by mitotic inhibitors. |
| In vitro generation is suppressed by the specific antibody (or probably by the antigen-antibody complex). |
| Nylon wool adherent. |
| Short-lived T cell (half life about 5 wk). |
| Non-recirculating T cell (resistant to or enriched by the in vivo administration of ALS). |
| Development is prohibited by the pretreatment with cyclophosphamide. |
| The latent period for induction is longer, compared with helper T cell, suppressor T cell, or DTH-mediating T cell. |

complicated. Thus, with the use of normal spleen cells as a B-cell source, helper activity was shown to develop as early as 3 days after immunization, conforming to the results by Araneo et al. (26). However, when hapten-primed B cells were used as a B-cell source, helper activity was not significantly detected in day 3 spleen cells, but found in day 7 spleen cells. The failure of day 3 spleen cells to express helper activity on the hapten-primed B cells was shown to be due to the concomitant existence of the suppressor T cells (Table VII, [27]). Thus, helper T-cell activity was able to be detected in day 3 spleen cells if the activity of suppressor T cells was eliminated by irradiation. Although it is not clarified why the activity of the suppressor T cells was manifested only when hapten-primed cells were used as a B-cell source, it seems most probable that memory B cells of high affinity antibody-producing cells are rich in hapten-primed spleen cells and the development of such cells to antibody-forming cells is preferentially suppressed by the effect of suppressor cells as shown by Takemori and Tada (28) in protein antigen system.

This and previous (10) studies showed that all of the helper, suppressor, and DTH-mediating T cells developed as early as 3 days after the immunization by 10^7 SRBC. Compared with the early appearance of these three types of functional T cells, the development of Tv was relatively delayed. Moreover it was shown that the failure of the generation of Ag-V-PFC in the culture of day 3 spleen cells may not be due to the existence of any suppressive influence (Table VIII). These results suggest that Tv belongs to the population of T cells different from the above three types of functional T cells. The requirement of longer period for the development after immunization might be the characteristic of cells in the T1 population. Thus, Araneo et al. (26) showed that a longer period was necessary for T1-enriched mice to express helper activity after immunization with SRBC than for T2-enriched mice.

The characteristics of Tv so far clarified in experiments using SRBC as the antigen are summarized in Table IX. It was thus demonstrated that VSV selectively replicated in the subpopulation of antigen reactive T cells. However, two questions remain. First, what is the function of the VSV-replicating T cell in the immune response?, and second, what is the cytological basis of selectivity of VSV-replication for the T-cell subpopulation? The first point will be analyzed in the accompanying paper (23).

Summary

Immunocytological properties of the splenic T cell (Tv) which develop into virus plaque-forming cells in response to the antigenic challenge in vitro were investigated
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in relation to the properties of helper T cells and suppressor T cells in antibody response. Tv was observed in spleen around 1 wk after the intravenous injection of mice with $10^7$ sheep erythrocytes. This contrasted with the finding that both helper T cells and suppressor T cells developed as early as 3 days after the immunization. Tv was proliferative in response to the antigenic stimulation, whereas helper T-cell activity could be expressed without cell division. Development of Tv to virus plaque-forming cells was much more dependent on macrophages than the generation of helper activity.

Tv was found in nylon wool adherent fraction, whereas helper T cell was found in both nylon adherent and nonadherent fractions. Tv belongs to the short-lived and nonrecirculating T-cell population (T1), whereas the major part of helper T cells belongs to the long-lived and recirculating T-cell population (T2). These results strongly suggest that vesicular stomatitis virus infect and replicate in the different subset(s) of T cell(s) to which the major part of helper T cells belong.

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