A T HELPER CELL FOR ANTI-VIRAL CYTOTOXIC T-CELL RESPONSES

BY R. B. ASHMAN AND A. MÜLLBACHER*

From the Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra City, A.C.T., Australia.

The generation in vitro of a cytotoxic response against allogeneic cells has been shown to require the collaboration of two T-cell types; a cytotoxic precursor expressing Ly 2, 3 antigens and a helper cell bearing the Ly 1 antigen (1). Pilarski has investigated the nature of this interaction, and has shown that there is an absolute requirement for radioresistant T helper (Th) cells in the generation of cytotoxic cells from thymocyte precursors. The Th cells are antigen specific but not strain specific (2) and belong to the Ly 1 subclass.

The role of Th cells in H-2-restricted cytotoxic T-cell (Tc cell) responses, however, has been less clearly delineated. A requirement for Ly 1+ Th cells that recognize self I region-coded determinants plus virus has been inferred from experiments both in vivo (3, 4) and in vitro (5, 6), but the existence of such cells has not yet been formally demonstrated. In this report we show that virus-specific radioresistant Th cells significantly augment the clonal expansion of H-2-restricted, anti-viral Tc cells in vitro.

Materials and Methods

Animals. Mice were bred at The John Curtin School of Medical Research, Australian National University, Canberra City, Australia. Only female mice, between 6 and 12 wk of age, were used in experiments.

Viruses and Immunization. Sendai virus and influenza virus strain A/JAP were grown and titrated as described previously (7, 8). BALB/c and CBA mice were immunized with either 10^6 EID_{50} of Sendai i.p., or 10^7 EID_{50} of influenza A/JAP i.p., and used for primed spleen populations 3–9 wk later.

Fractionation of Ig+ and Ig- Cells. Spleens were removed aseptically from primed or normal mice, dispersed by forcing through a wire grid, and washed once in Eagle's minimum essential medium supplemented with 10% fetal calf serum and 10^{-4} M 2-mercaptoethanol (MEM). The cell suspensions were separated into Ig+ and Ig- fractions as described by Parish et al. (9), washed twice and counted.

Antiserum Treatment. Approximately 1.5 × 10^7 Ig- primed spleen cells were treated with either a 1:5 dilution of anti-theta serum, a 1:3 dilution of anti-Ly 1.2, -Ly 2.2, or -Ly 3.2 serum (donated by Dr. I. F. C. McKenzie, University of Melbourne, Austin Hospital, Heidelberg, Australia) or a 1:3 dilution of anti Ia serum, in a final vol of 0.6 ml for 30 min at 0°C. Dilutions were made in MEM without serum. The cell suspensions were washed once, resuspended in 0.7 ml of a 1:6 dilution of low-toxicity rabbit complement, and incubated for 30 min at 37°C.

* Present address: Clinical Research Centre, Transplantation Biology Division, Harrow, Middlesex, England.

1 Al-Adra, A. R., J. M. Pilarski, and I. F. C. McKenzie. Surface markers on the T cells that regulate cytotoxic T-cell responses I. Distribution of Ly 1.1, Ly 2.1, Ly 5.1, and Ia* antigens on helper cells, suppressor cells, and cytotoxic cells. Submitted for publication.
Control spleen cell preparations were treated similarly. The cells were washed once more, and viability was determined by trypan blue exclusion.

**Depletion of Phagocytic Cells.** Erythrocytes and dead cells were removed from spleen cell suspensions by centrifugation on Ficoll-(Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) Isoopaque (sodium metrizoate, Nyegaard & Co., A/S, Oslo, Norway) gradients (10), the leucocytes were washed twice and were depleted of phagocytic cells by carbonyl iron treatment (11).

**In Vitro Generation of Virus-specific Tc Cells.** The method used was a modification of a limiting dilution assay. Normal spleen cells separated by centrifugation on Ficoll-Isoopaque gradients were used as both responder and stimulator cells. The latter were infected with virus at a multiplicity of infection of 1 EID\textsubscript{50} Sendai virus, or 1.5 EID\textsubscript{50} influenza A/JAP virus for 60 min at 37° C, and washed twice. Both stimulator cells and the cell preparations to be used as helper cells received 2,000 rad from a \textsuperscript{60}Co source. Appropriate numbers of responder, stimulator, and helper cells were dispensed, in a total vol of 0.1 ml, into 96-well, round-bottom Linbro tissue culture trays (Linbro Chemical Co., Hamden, Conn.), and incubated at 37°C for 5 d in a gas mixture consisting of 9.87% CO\textsubscript{2}, 6.92% O\textsubscript{2}, and 83.21% N\textsubscript{2}.

**Cytotoxicity Assay.** The trays were spun at 300 g for 5 min, the medium flicked off and replaced with 0.1 ml of fresh MEM. P815 mastocytoma cells that share H-2\textsuperscript{a} with the BALB/c Tc cells were labeled and infected as described previously (12) and \(1 \times 10\textsuperscript{4}\) target cells in 0.1 ml of medium added to each well. After a further 6-h incubation at 37°C, 0.1 ml of supernate was carefully removed from each well, the radioactivity was measured using a Packard Auto-Gamma spectrometer (model 5210, Packard Instrument Co., Downers Grove, Ill.), and \textsuperscript{51}Cr release from the target cells calculated as follows:

\[
\text{Percentage of lysis} = \frac{2 \times \text{Cr in supernate}}{\text{total releasable}} \times \times \text{100}. 
\]

The total releasable \textsuperscript{51}Cr was determined by lysing triplicate aliquots of target cells in water. Spontaneous release from targets in the presence of medium alone was assessed from six replicates on each tray. The mean ranged from 8 to 15% over the 6-hr assay period. The frequency distribution of the levels of lysis in wells assayed for cytotoxic activity was highly skewed, with some values >60%. Wells showing lysis >3 SD above the mean of the spontaneous release were scored as positive.

Control cultures consisted of irradiated primed spleen cells and infected irradiated stimulator cells, but without the normal responder cells. After 5 d of incubation individual wells were assayed for cytotoxic activity against infected target cells. The results were always negative, showing that cytotoxic activity originated only in the unprimed responder cells.

Experiments were usually repeated three times, but only one set of data representative of each series of experiments is shown in the Tables. Statistical significance was evaluated by arranging the data into 2 X 2 contingency tables and applying the chi-square test using Yates's correction for continuity.

**Results**

**Characteristics of the Helper Cell.** Spleen leucocytes from BALB/c mice primed with influenza A/JAP virus were subjected to various treatments, and were tested for their ability to help the anti-viral Tc-cell response of normal BALB/c spleen cells as measured by lysis of JAP-infected P815 target cells (Table I). Treatment with anti-theta serum and complement completely abolished the helper effect. Cell preparations enriched in Ig\textsuperscript{+} lymphocytes were significantly more effective than unfractioned cells, whereas the Ig\textsuperscript{-} fraction contained no helper activity. Depletion of phagocytic cells from the irradiated spleen cells did not affect the Tc-cell response. These results demonstrated that the helper cells were T cells.

**Ly and Ia Phenotype of the Helper Cell.** Treatment of an Ig\textsuperscript{-}-enriched primed spleen cell preparation with anti-Ly serum showed that these cells bore only the Ly 1 antigen (Table II).
TABLE I
Effect of Various Treatments on the Helper Activity of Primed Spleen Cells

| Treatment                      | Number of positive wells/total tested |
|-------------------------------|---------------------------------------|
| None                          | 18/45                                 |
| Anti-theta                    | 0/45§                                 |
| Ig+                           | 1/45§                                 |
| Ig-                           | 33/44§                                |
| Macrophage depletion          | 16/45                                 |

* Primed spleen cell suspensions were either treated with anti-theta serum and complement, or separated into Ig+ and Ig- fractions by rosetting, or depleted of phagocytic cells by carbonyl iron treatment, as described in Materials and Methods. The treated cells were then given 2,000 rad from a 60Co source, and 1 × 10^6 helper cells, together with 5 × 10^4 normal responder cells and 5 × 10^4 influenza A/JAP-infected stimulator cells dispensed into each well of a 96-well Linbro tissue-culture plate. The plates were incubated for 5 d and individual wells were scored as positive or negative for lytic activity against JAP-infected P815 targets. Control cultures consisting of helper cells and stimulator cells without responder cells showed no cytotoxic activity.

§ Significantly less than untreated controls, P < .001.

$ Significantly greater than untreated controls, P < .01.

TABLE II
Ly Phenotype of the Th Cell

| Treatment                      | Percentage of cells killed | Number positive wells/number tested |
|-------------------------------|---------------------------|-------------------------------------|
| Complement alone              | 16                        | 17/30                               |
| Anti-Ly 1.2 + Complement      | 83                        | 0/30§                               |
| Anti-Ly 2.2 + Complement      | 32                        | 18/30                               |
| Anti-Ly 3.2 + Complement      | 57                        | 18/30                               |

* Ig- spleen cells were separated from primed spleen cell suspensions by rosetting and treated with anti-Ly sera and complement as described in Materials and Methods. The treated cells were established in culture, at an original density (i.e., before treatment) of 2 × 10^4/well, with 5 × 10^4 normal responder cells, and 5 × 10^4 influenza A/JAP-infected stimulator cells. The cultures were incubated, harvested, and assayed as described above.

§ Significantly less than complement control, P < .001.

We also tested Th activity in Ig- cells depleted of Ia-positive cells. In a typical experiment, 3% of cells were killed by treatment with Ia antiserum and complement. Treated Th cells gave 13/28 positive wells compared to controls which showed 13/27 positive wells, indicating that the helper activity was mediated by an Ia-negative cell population.

**Helper Activity is Virus Specific.** To establish whether the Th cells were specific for different viruses, Ig- BALB/c spleen cells primed with either Sendai or influenza A/JAP viruses were cultured with normal BALB/c responder cells, and stimulated by syngeneic cells infected with either the homologous or heterologous virus. Table III shows clearly that the helper cells were activated only by stimulation with the virus with which the mice were primed.

**Help Can Be Delivered across an Allogeneic Barrier.** The need for compatibility between Th cells and Tc cells was tested by using either BALB/c or CBA Ig- cells from virus-primed or normal mice as helper cells for normal BALB/c responder cells stimulated with influenza A/JAP-infected BALB/c spleen cells. To control for possible cross-reactivity of BALB/c Tc cells against CBA (H-2k) on the P815 (DBA/2) tumor target cell line, cultures were also set up without syngeneic infected stimulator cells. Minimal Tc-cell activity was generated in these cultures (Table IV). Normal allogeneic cells
TABLE III

| Virus used for stimulation | Virus used for priming |
|---------------------------|-------------------------|
|                           | Influenza A/JAP         | Sendai       |
| Influenza A/JAP           | 27/29*                  | 6/30         |
| Sendai                    | 4/27                    | 25/29        |

Ig− helper cells were prepared as described, and 2 × 10⁶ cells/well used in the generation of responses against either the homologous or heterologous virus, using 5 × 10⁴ responders/well in the response against A/JAP and 7 × 10⁴ responders/well in the response against Sendai.

* Number of positive wells/total number tested. In both cases stimulation with the homologous virus gave a significantly greater number of positive wells (P < .001).

TABLE IV

| Stimulator cells | Helper cells* |
|------------------|---------------|
|                  | BALB/c        | CBA           |
|                  | Normal        | Primed        | Normal        | Primed        |
| None             | ND            | 1/30‡         | 2/30          |
| BALB/c           | 2/30          | 20/30         | 3/30          | 18/30         |

* Ig− cells were obtained from normal and primed BALB/c and CBA mice, and 2 × 10⁵ cells added to 5 × 10⁴ BALB/c responder cells in each well. The cultures were stimulated with 5 × 10⁴ A/JAP-infected BALB/c cells/well.

‡ Number of positive wells/total number tested. Primed cells showed significant enhancement of the cytotoxic response compared to normal cells (P < .001); however, there was no significant difference in the responses generated using helper cells from the two different strains. ND, not done.

did not enhance the response, thus excluding any allogeneic effect. Primed syngeneic and allogeneic helper cells both potentiated the cytotoxic response to approximately the same degree.

Discussion

This report formally demonstrates the existence of virus-specific Th cells that amplify the virus-specific Tc-cell response in vitro. As in allogeneic systems (1, 2), the helper cells carry the theta and Ly 1 membrane antigens.

A requirement for I region compatibility between Th and Tc cells has been postulated by Zinkernagel et al. (3); and von Boehmer et al. (4) have obtained results consistent with this prediction. These latter investigators have constructed a theoretical model that states, inter alia, that the generation of Tc cells requires participation of Th cells that are H-2 restricted: they recognize foreign antigens only in conjunction with self determinants coded for by genes in the I region of the major histocompatibility complex, and must also share I region alleles with the Tc-cell precursor. This model has a certain elegance, in that the requirements for Th-Tc cell interactions parallel those reported by Sprent (13, 14) for T-B cell collaboration, and if correct, would emphasize the symmetry between cellular and humoral immune responses in the induction of Th responses and the delivery of help signals. However, Bennink and Doherty (15) have shown that Tc cells against vaccinia virus do not need to share I region alleles with the recipient in which they are stimulated. Although there is no direct evidence that Th cells are required in this system, if they are involved apparently they operate without the need to recognize the same Ia antigen on both the stimulator cell and the Tc cells they help.
Clearly, the Th cells active in our system delivered help unimpeded by the difference between BALB/c (H-2<sup>d</sup>) and CBA (H-2<sup>k</sup>), but we do not know the precise mechanism of the helper effect. For example, there could have been sufficient numbers of potential helper cells in the BALB/c responding population to provide restricted help to BALB/c Tc cells (if such help is required), and the presence of the CBA Th cells could have allowed the BALB/c Th cells to express their full helper potential. Nevertheless, our results imply unrestricted interactions either between Th-Th cells or between Th-Tc cells. If this latter conclusion is correct, additional assumptions will have to be made to explain the apparent I region restriction of helper activity in vivo (3, 4).

Although our data (Table IV) could be interpreted as arguing against a role for self Ia antigens in the induction of Tc-cell responses, we do not favor this proposition. As precautions were not taken to confine infection strictly to the BALB/c stimulator cells, it is probable that cross-infection of CBA cells occurred in the cultures, so that the CBA Th cells could have been stimulated by syngeneic infected cells. Techniques to prevent cross-infection (11) could be applied to our system to rigorously test this question.

With the development of the in vitro assay for Th cells described here, the genetic and cellular requirements for the induction and delivery of help, and the role of Th cells in the generation of Tc-cell responses can be extensively investigated.

**Summary**

We demonstrate here: (a) the existence of T helper (Th) cells that augment the generation of virus-specific cytotoxic T cells in vitro, (b) that the helper cells carry the theta and Ly 1 membrane antigens, (c) that activation of the Th effect is specific for viral antigens, and (d) that the delivery of help is not H-2 restricted.

We thank Dr. C. R. Parish for the suggestion that led to the initiation of this study and for the provision of various antisera, and Dr. R. V. Blanden for helpful discussions and criticism of the manuscript.

*Received for publication 24 July 1979*

**References**

1. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens II. Cooperation between subclasses of Ly<sup>+</sup> cells in the generation of killer activity. *J. Exp. Med.* 141:1390.

2. Pilarski, L. M. 1977. A requirement for antigen-specific helper T cells in the generation of cytotoxic T cells from thymocyte precursors. *J. Exp. Med.* 145:709.

3. Zinkernagel, R. M., G. N. Callahan, A. Althage, S. Cooper, J. W. Streilein, and J. Klein. 1978. The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells: evidence for T help. *J. Exp. Med.* 147:897.

4. von Boehmer, H., W. Haas, and N. K. Jerne. 1978. Major histocompatibility complex-linked immune-responsiveness is acquired by lymphocytes of low-responder mice differentiating in thymus of high-responder mice. *Proc. Natl. Acad. Sci. U. S. A.* 75:2439.

5. Pang, T., I. F. C. McKenzie, and R. V. Blanden. 1976. Cooperation between mouse T cell subpopulations in the cell-mediated response to a natural poxvirus pathogen. *Cell. Immunol.* 23:153.

6. Pang, T., and R. V. Blanden. 1976. Genetic factors in the stimulation of T cell responses against ectromelia-infected cells: role of H-2K, H-2D and H-2I genes. *Aust. J. Exp. Biol. Med. Sci.* 55:549.
7. Doherty, P. C., and R. M. Zinkernagel. 1975. Specific immune lysis of paramyxovirus-infected cells by H-2-compatible thymus-derived lymphocytes. *Immunology*. 31:277.

8. Yap, K. L., and G. L. Ada. 1977. Cytotoxic T cells specific for influenza virus-infected target cells. *Immunology*. 32:151.

9. Parish, C. R., S. M. Kirov, N. Bowern, and R. V. Blanden. 1974. A one step procedure for separating mouse T and B lymphocytes. *Eur. J. Immunol.* 4:808.

10. Davidson, W. F., and C. R. Parish. 1975. A procedure for removing red cells and dead cells from lymphoid cell suspensions. *J. Immunol. Methods*. 7:291.

11. Pang, T., and R. V. Blanden. 1977. Requirements for stimulation of T cell responses against virus-infected cells: nature of ectromelia virus-infected cells capable of stimulating cytotoxic T cells in a secondary response *in vitro*. *Aust. J. Exp. Biol. Med. Sci.* 55:539.

12. Gardner, I. D., and R. V. Blanden. 1976. The cell mediated immune response to ectromelia virus infection II. Secondary response *in vitro* and kinetics of memory T cell production *in vivo*. *Cell. Immunol.* 22:283.

13. Sprent, J. 1978. Restricted helper function of F1 hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain mice. I. Failure to collaborate with B cells of the opposite parental strain not associated with active suppression. *J. Exp. Med.* 147:1142.

14. Sprent, J. 1978. Restricted helper function of F1 hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain mice II. Evidence for restrictions affecting helper cell induction and T-B collaboration, both mapping to the K-end of the H-2 complex. *J. Exp. Med.* 147:1159.

15. Bennink, J. R., and P. C. Doherty. 1978. Different rules govern help for cytotoxic T cells and B cells. *Nature (Lond.)*. 276:829.