

## B CELL ACTIVATION IN VIVO BY NONANTIGEN-SPECIFIC INTERACTION WITH T CELLS

### FREQUENCY OF IMMUNE RESPONSES INCREASED BY IMMUNIZATION WITH TWO ANTIGENS\*

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There is substantial evidence for a nonspecific mechanism of activation of thymus-independent bone marrow-derived precursors of immunocytes (B cells) in primary and secondary immune responses elicited in vitro (1-5) and in animals undergoing graft-*versus*-host reactions (6). Thymus-derived cells primed by immunogens other than the test antigens (not cross-reacting at the level of antibody-forming cells) may be utilized in such a mechanism (7). The common feature of most reported experiments is that preexposure of lymphoid cells to a priming antigen enhances the responsiveness of spleen cells to a second test antigen, provided that both immunogens are present at the time of the second immunization. It is generally presumed that the enhancing effect is due to proliferation of thymus-derived cells with helper function for antibody formation by B cells (including antigen-reactive cells and their activated progeny, i.e. inducer cells) (T cells) in response to the antigen administered first and to subsequent interaction of the activated thymic inducers with the subpopulations of B cells specific for the test antigens. It is plausible to propose that this type of T-B cell interaction is mediated by a diffusible nonantigen-specific product of activated T cells (1, 8).

We now report in vivo experiments describing nonspecific augmentation of murine immune responses using mixtures of two xenogeneic erythrocytes as antigens for limiting numbers of potentially competent cells. Our experiments were designed to determine whether the antigens could be administered simultaneously, and whether activation of T or B cells by the priming antigen was necessary for augmentation. For this purpose we determined the frequency and magnitude of anti-sheep erythrocyte responses by transferred bone marrow-thymus cell mixtures under conditions in which only one cell type was limiting. This approach originates from previous work, according to which the number of T or B cells activated during primary immune responses can be assessed by limiting dilution assays (9, 10).

The studies were carried out using a syngeneic cell transfer system in (C57BL/10 × C3H/He)F<sub>1</sub> female mice. The thymus of 6-8-wk old animals was the source of antigen-reactive

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helper cells (ARC), and the bone marrow of adult mice provided precursors of immunocytes. Details of the preparation of cell suspensions, the total body irradiation of recipients, the transplantation of cell mixtures, and the statistical procedures have been given elsewhere (9, 10). A total of  $5 \times 10^8$  washed erythrocytes were used for immunization, regardless of whether the red blood cells were from one or two species.

Groups of 10–14-wk old mice were exposed to X- or  $\gamma$ -irradiation and injected intravenously within a few hours with one of the following cell mixtures: (a) fixed number of  $1-3 \times 10^7$  bone marrow cells, providing an excess of immunocyte precursors, together with graded numbers of thymocytes providing limiting numbers of ARC. (b) Fixed number of  $5 \times 10^7$  thymocytes together with limiting numbers of bone marrow cells. All irradiated mice were immunized intravenously 1 day later. The response was assessed in terms of the number of antibody-forming cells secreting  $\gamma$ M hemolysins, i.e. direct plaque-forming cells (PFC), in the spleens of recipients at the time of peak response.

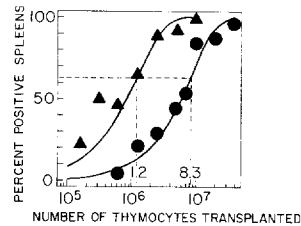


FIG. 1. Percentage of recipient mice with spleens positive for anti-sheep PFC 8 days after transplantation of  $13-15 \times 10^6$  bone marrow cells and limiting numbers of thymocytes. Symbols indicate observed percentages after immunization against SRBC alone (●) or against SRBC + HRBC (▲), and the fitted curves expected percentages according to the Poisson model. The probabilities of positive spleens for  $10^6$  transplanted thymocytes (and 95% confidence intervals) were 0.12 (0.09–0.15) and 0.80 (0.58–1.10), respectively. Dashed straight lines indicate the number of thymocytes containing an average of one detectable ARC:  $8.33 \times 10^6$  (6.25–10.42) thymocytes for the SRBC immunization plot, and  $1.25 \times 10^6$  (0.91–1.72) for the SRBC + HRBC immunization plot.

The antigens were sheep, chicken, burro, or horse erythrocytes (SRBC, CRBC, BRBC, and HRBC, respectively) in different combinations. These antigens were chosen because previous experiments demonstrated that the direct PFC which arose in the spleens of irradiated recipients of nonlimiting numbers of marrow-thymus cells in response to horse, burro, and chicken erythrocytes had little, if any, cross-reactivity with SRBC.

The frequencies of thymic ARC available for activation by SRBC alone or by a mixture of SRBC and HRBC were determined by a limiting dilution experiment. The mice were irradiated (925 R) and injected with  $1.3-1.5 \times 10^7$  marrow cells together with graded numbers of thymocytes, in a range extending from 0.16 to  $40 \times 10^6$  cells, as indicated in Fig. 1. One day after transplantation, each recipient was given either  $5 \times 10^8$  SRBC or a mixture of  $2.5 \times 10^8$  SRBC with an equal number of HRBC. A total of 204 mice received SRBC, and 97 mice SRBC + HRBC. Each thymocyte inoculum was given to at least 10 mice. Direct PFC of recipient spleens were enumerated 8 days after transplantation, and spleens were regarded as positive if the number of PFC was larger than 150.

The proportion of mice with positive spleens increased as the number of transplanted thymocytes was raised from  $6.2 \times 10^5$  to  $4 \times 10^7$  in the SRBC

immunization series. The relationship between the number of thymocytes and the proportion of responding animals conformed to the predictions of the Poisson model. The estimated concentration of activated ARC (calculated by the method of maximum likelihood) was 0.12 in  $10^6$  (see legend of Fig. 1 for confidence intervals), or one ARC in  $8.3 \times 10^6$  thymocytes. The results obtained in the SRBC + HRBC immunization series also conformed to the Poisson model. However, the dilution curve was shifted to the left as the frequency of anti-sheep responses increased over the range of  $1.6 \times 10^5$ – $1 \times 10^7$  transplanted thymocytes (Fig. 1). The concentration of ARC detectable in this

TABLE I  
*Frequency of Anti-Sheep and Anti-Burro PFC Responses in Spleens of Irradiated-Reconstituted Mice after Immunization with Two Antigen Complexes*

No. of cells transplanted*		Immunization	Positive spleens for anti-SRBC PFC†			Positive spleens for anti-BRBC PFC†		
Bone marrow ( $\times 10^6$ )	Thymocytes ( $\times 10^6$ )		Fraction	Per cent $\pm$ SE	PFC/spleen‡	Fraction	Per cent $\pm$ SE	PFC/spleen‡
13-15	—	SRBC + HRBC	0/10					
13-15	—	SRBC + CRBC	0/20					
30	—	BRBC				0/5		
30	—	SRBC + BRBC	1/5		355	0/5		
13-15	0.62	SRBC	1/10	10 $\pm$ 9	218			
30	0.62	BRBC				4/10	40 $\pm$ 15	251 $\pm$ 39
13-15	0.62	SRBC + HRBC	10/21	48 $\pm$ 11	337 $\pm$ 170			
30	0.62	SRBC + BRBC	10/11	91 $\pm$ 8	487 $\pm$ 132	8/11	73 $\pm$ 13	309 $\pm$ 70
13-15	0.62	SRBC + CRBC	8/25	32 $\pm$ 9	223 $\pm$ 67			
0.03	50	SRBC	13/26	50 $\pm$ 10	282 $\pm$ 30			
0.03	50	SRBC + HRBC	8/23	35 $\pm$ 10	337 $\pm$ 50			
0.03	50	SRBC + CRBC	9/22	41 $\pm$ 11	333 $\pm$ 90			

\* After 925 R of X-rays or 950 rads of  $^{137}\text{Cs}$   $\gamma$  rays.

† Spleens with more than 200 direct PFC.

‡ Geometric mean  $\pm$  standard error.

second series was  $\sim 7$  times higher than after immunization with SRBC alone, i.e., it was 0.8 in  $10^6$  or one ARC in  $1.2 \times 10^6$  thymocytes. Despite the increased frequency of responses, the mean number of PFC per positive spleen remained comparable in the two experimental series, ranging from 300 to 800. Thus, the immunization with HRBC and SRBC increased the number of activated ARC detected by interaction with SRBC-specific precursors and production of anti-sheep PFC.

We determined the effect of other combinations of xenogeneic erythrocytes on the frequency of anti-sheep and anti-burro PFC responses. Three sets of data are presented in Table I. First, irradiated control mice reconstituted with bone marrow cells alone failed to form significant numbers of PFC even upon immunization with erythrocyte mixtures (upper section of Table I). Second,

the proportion of responding animals increased both for anti-sheep and anti-burro PFC upon immunization of irradiated mice with the combinations SRBC + BRBC and SRBC + CRBC, provided that the number of reconstituting thymocytes, but not of marrow cells, was limiting (middle section of Table I). Third, the proportion of responding animals did not increase for anti-sheep PFC upon immunization of irradiated mice with erythrocyte mixtures if only the number of reconstituting marrow cells was limiting (lower section of Table I).

The data show that the simultaneous stimulation of relatively small lymphoid cell populations by two weakly or noncross-reacting antigens resulted in a severalfold increase of the number of functional antigen-sensitive units. By limiting one of the cell types constituting these units of immune response, it was established that the effect was elicited exclusively at the level of T cells.

Two explanations can be offered for the increased activation of thymic ARC by antigen mixtures under these experimental conditions. The antigens may have synergized in the triggering of specific ARC by promoting some form of T-T cell interaction (11) or by removing repressive functions (12), thus lowering the threshold for activation. However, the number of specific ARC activated by some of the priming antigens may have been considerably larger than that of SRBC-specific ARC.<sup>1</sup> Should T-B cell interactions be mediated by nonantigen-specific T-cell products (1, 8), then our results could be explained by differential "strength" of the admixed antigens reflected in the number of ARC activated. The two proposed explanations are not mutually exclusive. We consider it unlikely that these data can be accounted for simply by cross-reactions at the T-cell level (7) since cross-activation of SRBC-specific B cells was not achieved with BRBC or HRBC-educated T cells in two-step experiments (9, 13).

An important aspect of our experiments is the apparently common occurrence in vivo, without the necessity of graft-*versus*-host reactions, of a kind of T-B cell interaction (via nonspecific mediators) not requiring associated recognition of carrier and hapten determinants by T and B or by macrophage and B cells (via membrane-bound receptors). Most likely, our findings are a simplified example of adjuvant effects recently associated with T-cell functions (14, 15). The emerging picture is one of the thymus assuming the major role of modulating the immune response by a variety of mechanisms.

#### SUMMARY

The number of direct ( $\gamma$ M) hemolytic plaque responses of irradiated mice, repopulated with relatively small and limiting numbers of bone marrow and thymus cells, was increased by the simultaneous immunization with two antigen

<sup>1</sup> Trizio, D., and G. Cudkowicz. Unpublished data for burro-specific ARC of (C57BL/10  $\times$  C3H)F<sub>1</sub> mice.

complexes instead of one. Anti-sheep responses were augmented by the following antigen combinations: SRBC + HRBC, SRBC + BRBC, and SRBC + CRBC. Limiting either thymocytes or bone marrow cells indicated that the antigen mixtures acted at the level of T cells increasing severalfold the number of triggered antigen-reactive cells. It was concluded that one of the antigens could have influenced the triggering of antigen-reactive cells specific for the other by promoting synergistic or derepressive T-T cell interactions. Moreover, bone marrow precursor cells could have been activated by the thymic inducers specific for the test antigens as well as by those specific for the second of the priming antigens.

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