THE ROLE OF H-2-LINKED GENES IN HELPER T CELL FUNCTION

VII. Expression of *I* Region and Immune Response Genes by B Cells in Bystander Help Assays*

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For some years we and others have been interested in the mode of action of what have come to be termed bystander or nonspecific helper T cells. These are T cells primed to a particular antigen that, in the presence of that antigen, help the response of B cells to determinants linked to some other (indicator) antigen, such as sheep erythrocytes (SRBC).[1] This helper activity is shown to be a specific response by the T cells to the antigen with which they were primed, and not the result of some cross-reaction between the priming and indicator antigens, as omission of the priming antigen from the cultures results in profound loss of helper activity (1–3). An additional piece of evidence to support this conclusion is that a number of different antigens, keyhole limpet hemocyanin (KLH), horse erythrocytes (HRBC), human gamma globulin (HgG), alloantigens, and fowl gamma globulin (FgG), to name but a few, have been used to elicit bystander help (1–6), and it is hard to imagine that all these antigens would cross-react in mice with SRBC.

Because helper T cells that respond to a variety of different antigens could promote the response of bystander B cells to erythrocyte (RBC)-bound determinants it was thought that bystander help was mediated by nonspecific factors such as those found in the supernates of T cells responding to concanavalin A or in mixed lymphocyte reactions. Indeed the supernate of antigen-primed T cells that respond to the priming antigen has been shown to contain such factors (6–9). In support of this theory is the observation that bystander help is relatively easy to generate for B cells that respond to RBC as the indicator antigen, but harder to demonstrate for responses to protein-bound antigens, especially in the absence of T cells specific for that protein (4, 10). Supernates from activated T cells are likewise very active in stimulating anti-RBC

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[1] *Abbreviations used in this paper:* (B6A)F_1, (C57BL/6 × A/J)F_1; B10, C57BL/10.SgSn; BSS, balanced salt solution; CFA, complete Freund's adjuvant; FgG, fowl gamma globulin; HgG, human gamma globulin; HRBC, horse erythrocyte(s); KLH, keyhole limpet hemocyanin; MØ, macrophage; PFC, plaque-forming cells; RBC, erythrocytes; SRBC, sheep erythrocyte(s); TCGF, T cell growth factor; (TG)-A--L, poly-L-(Tyr, Glu)-poly-D,L-Ala--poly-L-Lys; TNP, trinitrophenylated; TRF, thymus replacing factor.

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responses, but only rarely and under special circumstances stimulate responses to other antigens such as trinitrophenylated (TNP)-KLH or TNP-FgG (7–9, 11).

Given these facts, a straightforward explanation of bystander help is that antigen-specific helper T cells recognize the antigen on the surface of suitable antigen-presenting cells, perhaps macrophages (MØ), in vitro, and in response secrete helper factors such as thymus replacing factor (TRF) and B cell growth factor or T cell growth factor (TCGF). These factors then diffuse in the culture medium to interact with bystander B cells with the potential to respond to RBC antigens. B cells responding to bystander protein antigens would not be helped by such activities because TRF-like factors are not usually sufficient for their response. If this model were correct, one would expect that bystander helper T cells would be controlled by immune response (*Ir*) and *I*-region genes expressed by accessory cells in the cultures, not necessarily by the B cells themselves. The experiments in this paper show that this prediction is not borne out and, under conditions of limiting bystander help, that this activity is controlled at least by *I* region and *Ir* genes expressed by B cells. The *Ir* genes in this case are specific for the priming antigen, even though the B cell is responding to some unrelated RBC. One explanation for these results is that B cells can take up antigen nonspecifically, and then display this antigen to helper T cells in association with products of the major histocompatibility complex in a fashion analogous to antigen-presenting MØ.

Materials and Methods

Animals. C57BL/6, C57BL/10.SgSn (B10), B10.A, and (C57BL/6 × A/J)F_1 (B6A)F_1 female mice were all purchased from The Jackson Laboratory, Bar Harbor, Maine. (B10.A × DBA/2)F_1 animals were bred in our own colony.

Preparation of Irradiated Bone Marrow-reconstituted Mice. Bone marrow chimeric mice were prepared as previously described (12). To summarize the methods used, both donors and recipients of bone marrow were given 0.04 ml rabbit anti-mouse thymocyte serum (Microbiological Associates, Walkersville, Md.) intraperitoneally 2 d before sacrifice or irradiation to deplete the animals of recirculating T cells (13). After receiving 950 rad from a ^{60}Co source, recipient mice were given 1–2 × 10^7 donor bone marrow cells intravenously. They were protected from infection by injection of 400 μg gentamicin sulfate (Schering Corp., Kenilworth, N. J.) intraperitoneally on the day before, day of, and day after irradiation, and by both acidification and chlorination of their drinking water. These chimeric animals were not used in experiments until at least 8 wk after irradiation. In referring to chimeric animals in this paper, an arrow (→) is used to designate the administration of bone marrow to an irradiated mouse. For example, (B6A)F_1 → B10.A represents an irradiated B10.A mouse reconstituted with (B6A)F_1 bone marrow.

Antigens. SRBC and HRBC were obtained from single animals at Colorado Serum Co., Denver, Colo. KLH was purchased from Calbiochem Behring Corp., American Hoechst Corp., San Diego, Calif. Poly-L-(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys [(TG)-A--L; batch numbers MC 6, 8, 9, and 10] was bought from Miles Laboratories, Inc., Elkhart, Ind.

Immunizations. Helper T cells were primed in vivo with (TG)-A--L by injection of 100 μg of the antigen in complete Freund's adjuvant (CFA) in the base of the tail. T cells were harvested 6–8 d later from the inguinal and periaortic lymph nodes of animals so injected (14, 15). Helper T cells were primed with KLH in a similar fashion, or by injection of 20–100 μg of KLH in CFA intraperitoneally into mice (3). In the latter protocol, T cells were obtained from the spleens of mice so injected 6–8 d later.

Preparation of Cells for Tissue Culture. T cells were purified by passage of cell suspensions over nylon fiber columns (16). B cell and MØ suspensions were obtained from spleens by treatment of spleen cell suspensions with a rabbit anti-T cell serum and guinea pig complement (3)

and/or with a hybridoma anti-Thy-1 reagent (T24/40.7; given to us by Dr. Ian Trowbridge, Salk Institute, San Diego, Calif.) and rabbit complement.

Antigen-presenting MØ activity was provided to cultures included in the preparations of splenic B cells. Additional MØ activity was added in some experiments by means of nonirradiated or irradiated (1,300 rad) anti-T plus complement-treated spleen cells or by normal peritoneal washings.

In certain experiments, splenic B cell preparations were passed through Sephadex G-10 columns by the method of Ly and Mishell (17). This procedure depleted the cells of antigen-presenting MØ, such that the cultures were no longer antigen responsive in the presence of 2-mercaptoethanol, and also may have removed a subpopulation of B cells.

Culture Conditions. Cells were cultured by modifications (18) of the methods of Mishell and Dutton (19) in Linbro FB 16-24TC culture trays (Linbro Chemical Company, Hamden, Conn.). Identical triplicate cultures were set up. Antigens were added to cultures in the following fashions: SRBC at 0.01–0.05%; (TG)-A--L and KLH added as soluble antigens at usual final concentrations of 100 and 5 μg/ml, respectively.

Quantitation of Helper Activity. Bystander helper activity was measured by titrating the T cells to be assayed into cultures that contained constant numbers of B cells, MØ, and constant amounts of SRBC and specific antigen, KLH, or (TG)-A--L (3). After 4 d, plaque-forming cells (PFC)/culture against the SRBC were measured and plotted against the number of T cells added to cultures. A straight line was fit to the initial linear portion of this titration, and the slope of the line taken as a relative measure of the helper activity of the T cell preparation expressed as PFC/10^6 T cells/culture ± SE. To control for any SRBC-specific helper activity in the T cell preparations, control titrations were always performed in which the specific antigen, KLH or (TG)-A--L, was omitted from the cultures.

PFC Assay. After 4 d, identical cultures were harvested and assayed for direct PFC against the appropriate antigen, SRBC, or in some cases HRBC, using the slide modification of the hemolytic plaque assay (19).

In some experiments that contained mixed *H-2*^{a} and *H-2*^{b} B cells, it was necessary to establish the *H-2* haplotype of the PFC. The relative contributions of different B cells to these total numbers was then established by treatment with the appropriate anti-*H-2* antisera plus complement followed by PFC determination. To accomplish this, triplicate cultures were resuspended to 1.6 ml in balanced salt solution (BSS). Total PFC/culture were established in the usual way (see above). Simultaneously, 0.4-ml aliquots were treated at 4°C for 30 min with: 1:10 dilution of the concentrated supernate of an anti-K^k hybridoma, 11.4.1, kindly given to us by the laboratory of Doctors L. A. and L. A. Herzenberg (20); or a 1:5 dilution of a (B10.A × DBA/2)F_1 anti-B10 anti-serum; or nothing. After 30 min, the aliquots were washed with BSS and incubated with 0.4 ml/aliquot of 1:15 rabbit complement in culture medium without fetal calf serum, under 10% CO_2 90% air, at 37°C for 1 h. The aliquots were then spun, supernate discarded, the pellets resuspended in ~0.2 ml BSS, and the entire contents of each aliquot assayed on two slides for PFC against SRBC. The proportions of PFC of each haplotype in the culture could then be established by calculation from the numbers of PFC destroyed by killing with anti-K^k plus complement or anti-*H-2*^{b} plus complement (21). During this procedures, between 50 and 80% of the PFC were lost nonspecifically in the controls, presumably as a result of the losses during washing and complement treatment. Nevertheless, reproducible measurements of the *H-2*-type of the PFC could be obtained.

Similar procedures, without the anti *H-2*^{a} treatment, were used in cultures that contained mixed nonirradiated *H-2*^{a} and (*H-2*^{b} × *H-2*^{a})F_1 B cells.

Results

(TG)-A--L-Driven Bystander Helper Activity. In previous experiments, we and others showed that T cells primed to protein antigens such as KLH or HgG helped B cells respond to bystander RBC-bound antigens such as SRBC, provided both the priming and RBC antigens were added to the cultures. One interesting and perhaps indicative feature of these responses was that high concentrations of the priming antigen in vitro were required to drive them (3, 4, 7, 22). We decided to investigate if bystander helper

activity could be demonstrated in (TG)-A--L-primed T cells, and if this activity was dependent upon high (TG)-A--L concentrations in vitro. T cells were therefore obtained from (TG)-A--L- or CFA-primed animals and titrated into cultures that contained syngeneic B cells, MØ, SRBC, and various concentrations of (TG)-A--L. For this experiment, C57Bl/6, *H-2*^{b}, mice were used, which are high responders to (TG)-A--L. The results of this experiment, shown in Table I, demonstrated that (TG)-A--L-driven bystander helper activity was indeed observed, but only at the highest concentration of (TG)-A--L used, 100 μg/ml. This phenomenon was dependent upon the priming of the T cells with (TG)-A--L because CFA-primed T cells did not stimulate such a response. In addition, the bystander activity was extraordinarily potent at stimulating large numbers of anti-SRBC PFC, an observation already made for other protein antigens (3, 4, 7, 21).

Immune Response Genes Affect Bystander Helper Activity. To investigate if *Ir* genes affect bystander help, (TG)-A--L-specific T cells were primed in (B6A)F_1 animals, the cross between C57Bl/6, *H-2*^{b}, high-responder animals, and A/J, *H-2*^{a}, low-responder animals. These T cells were then titrated for bystander helper activity in anti-SRBC responses of B10, *H-2*^{b}, or B10.A, *H-2*^{a}, B cells and MØ. As shown in Table II, these T cells stimulated a high (TG)-A--L-driven anti-SRBC response in cultures that contained high-responder B cells and MØ, but not in cultures of low-responder B cells and MØ. This response was dependent on the presence of (TG)-A--L in the cultures. In contrast, a good anti-SRBC response was obtained in cultures that contained either B10 or B10.A B cells and MØ, KLH-primed T cells, and KLH. Our conclusion was that *Ir* genes expressed by B cells and/or MØ functioned in the presentation of (TG)-A--L to antigen-primed T cells, even when these T cells were driving a bystander SRBC response.

Expression of (TG)-A--L-specific Ir Genes by B Cells Responding to SRBC. We then performed a series of experiments to test the hypothesis that the failure of (TG)-A--L-primed F_1 T cells to mediate a bystander response to SRBC with nonresponder B10.A B cells was the result of the failure of the B10.A T cell-depleted spleen cells to effectively present (TG)-A--L for the production of nonspecific helper factors.

Our first approach was to add back to these cultures known sources of high-responder antigen-presenting cells in an effort to stimulate the response of the B10.A B cells. In our initial experiments, we used peritoneal cells and irradiated T cell-depleted spleen as the source of antigen-presenting cells, both of which previously had been shown by us to be functional in this culture system (15, 18).

The results with peritoneal cells are shown in Table II. The addition of high

TABLE I

Antigen Concentration Dependence by Bystander Help

T cell priming	Antigens in culture	Bystander helper activity
(TG)-A--L	SRBC	2,110 ± 310
(TG)-A--L	1 μg/ml (TG)-A--L + SRBC	3,470 ± 510
(TG)-A--L	10 μg/ml (TG)-A--L + SRBC	3,200 ± 570
(TG)-A--L	100 μg/ml (TG)-A--L + SRBC	11,560 ± 1,120
CFA	100 μg/ml (TG)-A--L + SRBC	3,280 ± 420

* Anti-SRBC PFC/10^6 T cells per culture ± SE.

TABLE II

Expression of Ir Genes in Bystander Help Assays

Cells in culture		(B6A)F_1 T cell priming	Antigens in culture	Bystander helper activity‡
Splenic non-T	peritoneal washings*			
B10	B10	(TG)-A--L	SRBC + (TG)-A--L§	11,860 ± 2,280
B10.A	B10.A	(TG)-A--L	SRBC + (TG)-A--L	1,510 ± 342
B10	B10	(TG)-A--L	SRBC	3,910 ± 440
B10.A	B10.A	(TG)-A--L	SRBC	2,240 ± 380
B10	B10.A	(TG)-A--L	SRBC + (TG)-A--L	13,020 ± 1,530
B10.A	B10	(TG)-A--L	SRBC + (TG)-A--L	1,430 ± 200
B10	B10.A	(TG)-A--L	SRBC	1,020 ± 190
B10.A	B10	(TG)-A--L	SRBC	1,890 ± 384
B10	B10	KLH	SRBC + KLH‖	18,250 ± 1,020
B10.A	B10.A	KLH	SRBC + KLH	16,050 ± 2,370

* 10^5 cells from washings of normal animals/culture.
‡ Anti-SRBC PFC/10^6 T cells per culture ± SE.
§ 100 μg (TG)-A--L/ml.
‖ 5 μg KLH/ml.

TABLE III

Ir Genes Are Expressed at Least by B Cells in Bystander Helper Assays

Splenic non-T cells in culture	(B6A)F_1T cell priming	Antigens in culture	Bystander helper activity*
B10.A‡	(TG)-A--L	SRBC + (TG)-A--L§	10 ± 20
(B6A)F_1‡	(TG)-A--L	SRBC + (TG)-A--L	4,530 ± 1,070
Irradiated (B6A)F_1‡	(TG)-A--L	SRBC + (TG)-A--L	30 ± 10
B10.A + irradiated (B6A)F_1‖	(TG)-A--L	SRBC + (TG)-A--L	80 ± 40
B10.A‡	(TG)-A--L	SRBC	0 ± 90
(B6A)F_1‡	(TG)-A--L	SRBC	400 ± 140
B10.A‡	KLH	SRBC + KLH¶	2,850 ± 10
B10.A + irradiated (B6A)F_1‖	KLH	SRBC + KLH	1,220 ± 170

* Anti-SRBC PFC/10^6 T cells per culture + SE.
‡ 4×10^6/culture.
§ 100 μg/ml.
‖ 2×10^6 each/culture.
¶ 5 μg/ml.

responder B10 normal peritoneal MØ had no effect on the low anti-SRBC response of B10.A B cells, driven by (TG)-A--L primed F_1 T cells and (TG)-A--L, suggesting the surprising conclusion that *Ir* genes in these cultures were being expressed at least by B cells.

Similar results were obtained with irradiated T cell-depleted spleen cells and are shown in Table III. B10.A cells were not stimulated to respond to (TG)-A--L, even in the presence of equal numbers of irradiated (1,300 rad) (B6A)F_1 splenic non-T cells, a potent source of *H-2*-restricted antigen-presenting activity (23). Control experiments showed that the (B6A)F_1 cells, when not irradiated, would respond to SRBC driven

by helper cells sensitive to (TG)-A--L, and that the B10.A cells would respond to SRBC in the presence of bystander helper driven by KLH. In addition, by the use of F_1 cells as the source of antigen-presenting activity in this experiment, we eliminated the possibility of *H-2* restrictions in B cell/MØ interactions as the cause of the low response of the B10.A cells. Therefore, again we concluded that B cells at least were expressing *Ir*-genes in this bystander response.

Although we had previously demonstrated the antigen-presenting capabilities of peritoneal cells and irradiated T-depleted spleen cells (15, 18), we performed additional experiments with adherent cell-depleted B cells to further test our conclusions. To do this, B10.A and B10 splenic non-T cells were depleted of adherent cells by passage through Sephadex G-10 columns (17). We then tested the ability of these cells to respond to SRBC in the presence of (TG)-A--L-primed or KLH-primed (B6A)F_1 T cells and (TG)-A--L or KLH, respectively. Cultures were performed in the presence or absence of (B6A)F_1 peritoneal washing cells or irradiated (1,300 rad) (B6A)F_1 splenic non-T cells. One of the three experiments we have done is shown in Table IV. B10.A and B10 Sephadex G-10-passed B cells did not respond to SRBC in the presence of antigen and antigen-primed T cells in the absence of an added source of MØ. (B6A)F_1 peritoneal washings or irradiated splenic non-T cells restored the responses to SRBC driven by KLH-primed T cells plus KLH of both B10.A and B10 B cells equally. By contrast, in the presence of (TG)-A--L-primed T cells and (TG)-A--L, the responses to SRBC of B10 B cells were restored by MØ-sources much more successfully than those of B10.A B cells.

This experiment confirmed the functional capabilities of the peritoneal and irradiated spleen cells and supported our previous conclusion that B cells were expressing *Ir*-genes in this bystander response. It should be noted, however, that with this particular protocol, although the response of the high responder B cells was clearly

TABLE IV

Reconstitution of MØ-depleted B Cell Preparations

G10-passed B cells in culture	Source of antigen-presenting (B6A)F_1 cells	(B6A)F_1 T cell priming	Antigens in culture	Bystander helper activity*
B10.A	—	(TG)-A--L	SRBC + (TG)-A-L‡	−8 ± 7
B10	—	(TG)-A--L	SRBC + (TG)-A--L	20 ± 40
B10.A	Peritoneal washings§	(TG)-A--L	SRBC + (TG)-A--L	600 ± 80
B10	Peritoneal washings§	(TG)-A--L	SRBC + (TG)-A--L	2,570 ± 410
B10.A	Irradiated splenic non-T¶	(TG)-A--L	SRBC + (TG)-A--L	940 ± 310
B10	Irradiated splenic non-T¶	(TG)-A--L	SRBC + (TG)-A--L	5,480 ± 520
B10.A	—	KLH	SRBC + KLH‖	0 ± 0
B10	—	KLH	SRBC + KLH	−30 ± 30
B10.A	Peritoneal washings§	KLH	SRBC + KLH	1,930 ± 260
B10	Peritoneal washings§	KLH	SRBC + KLH	1,800 ± 24
B10.A	Irradiated splenic non-T¶	KLH	SRBC + KLH	1,450 ± 160
B10	Irradiated splenic non-T¶	KLH	SRBC + KLH	1,600 ± 210

* Anti-SRBC PFC/10^6 T cells per culture ± SE.

‡ 100 μg/ml.

§ 10^5 peritoneal washings/culture.

‖ 5 μg/ml.

¶ 1.5×10^6 irradiated splenic non-T + 1.5×10^6 B10.A or B10 B cells. All other cultures contained 3×10^6 B cells.

greater, some significant response was stimulated in the low responder B cells. This point is discussed more fully below.

One problem in interpreting the results shown in Tables II, III, and IV is that, regardless of the functional capabilities demonstrable in peritoneal cells and irradiated T cell-depleted spleen, an accessory cell necessary for the activation of the (TG)-A--L T cells may have been nevertheless absent in these populations. Because the exact nature of the antigen-presenting cell in spleen still remains somewhat in doubt, this is perhaps not a trivial objection. To meet this objection we decided to perform experiments in which preparations of T-depleted splenic cells from high and low responder animals were mixed. (TG)-A--L-primed (B6A)F_1 T cells were then titrated into these cultures in the presence of SRBC and (TG)-A--L. The mixture was incubated for 4 d and then assayed for PFC to SRBC. At the time of assay, the PFC were typed with anti-*H-2* sera and complement to establish the haplotype of the responding B cells. By this procedure, we provided full high-responder, splenic accessory cell, antigen-presenting activity to cultures that contained B cells from both high- and low-responder animals, and yet could still test which types of B cells responded in the cultures.

An experiment of this type is shown in Table V. B10 high-responder B cells responded very well to SRBC, helped by (B6A)F_1 T cells that responded to (TG)-A--L. As before, B10.A B cells did not. This result was unaffected by mixing the two types of splenic non-T cells together at the beginning of the culture period. B10.A B cells failed to receive (TG)-A--L-specific bystander help even in the presence of a full complement of B10 high-responder splenic non-T cells. A control experiment with the same mixtures of splenic non-T cells, KLH, and KLH-primed (B6A)F_1 T cells showed that the B10.A B cells could be triggered to give as large a SRBC-specific bystander response as the B10 B cells, using an antigen not under *Ir* gene control. As before, the implications of this experiment were that SRBC-specific B cells, at least, expressed (TG)-A--L-specific *Ir* genes when the anti-SRBC response was being driven by (TG)-A--L plus (TG)-A--L-specific T cells.

This type of experiment was also performed using a mixture of B10.A and (B6A)F_1

TABLE V

Ir Gene Expression by B Cells in Cultures that Contained both Responder and Nonresponder B Cells

Splenic non-T cells in culture	(B6A)F_1 T cell priming	Antigens in culture	Bystander helper activity*		
			Whole culture	B10 B cells	B10.A B cells
B10‡	(TG)-A--L	SRBC + (TG)-A--L§	3,675 ± 1,599		
B10.A‡	(TG)-A--L	SRBC + (TG)-A--L	25 ± 69		
B10 + B10.A‖	(TG)-A--L	SRBC + (TG)-A--L	3,720 ± 400	3,270	450
B10‡	(TG)-A--L	SRBC	0 ± 0		
B10.A‡	(TG)-A--L	SRBC	0 ± 0		
B10 + B10.A‖	(TG)-A--L	SRBC	0 ± 0	0	0
B10 + B10.A‖	KLH	SRBC + KLH¶	21,580 ± 1,330	11,220	10,360

* Anti-SRBC PFC/10^6 T cells/culture ± SE.
‡ 4×10^6/culture.
§ 100 μg/ml.
‖ 2×10^6 each/culture.
¶ 5 μg/ml.

T-depleted spleen cells (Table VI). As before, KLH-primed T cells stimulated an equivalent response in both B cells, but (TG)-A--L-primed T cells produced a much larger anti-SRBC response in (B6A)F_1 B cells than in B10.A B cells, again indicating the expression of *Ir*-genes in the SRBC responsive B cells. However, as in Table IV it should be noted that, with this particular protocol, some stimulation of the nonresponder cells was seen. This point is discussed more fully below.

I Region Genes on B Cells Control Bystander Helper T Cells. We wondered if the phenomenon we have observed was specific for *Ir* genes, or whether it could be extended to include antigens not under *Ir* gene control. To investigate this, (B6A)F_1 T cells were restricted for interaction in B10.A-irradiated mice [(B6A)F_1 → B10.A chimeric mice]. These T cells and control (B6A)F_1 T cells were primed with KLH and tritiated for their ability to help anti-SRBC responses of B10.A and B10 splenic B cells and MØ in the presence and absence of KLH. As shown in Table VII, bystander help from (B6A)F_1 → B10.A T cells was only apparent for B10.A B cells. B10 B cells were not helped, even when (B6A)F_1 peritoneal cells were added to the cultures. We have obtained similar results using irradiated (1,300 rad) (B6A)F_1 splenic non-T cells as additional accessory cells.

Thus, helper T cells specific for KLH appeared to be restricted by *I* region genes expressed at least by the B cells, even in these cultures in which responses to SRBC were being measured. This conclusion is analogous to that reached above for *Ir* gene expression in bystander responses.

Discussion

The experiments described in this paper answered a number of questions regarding the mechanisms of bystander helper T cells and also raised a number of questions concerning the mode of action and mechanisms of antigen recognition during T cell-B cell collaboration in linked antigen responses.

First, our experiments clearly showed that *Ir* genes control the action of bystander helper T cells. Thus (responder × nonresponder)F_1 (TG)-A--L-primed T cells could

TABLE VI

Ir Gene Expression by B Cells in Cultures that Contained both (Responder × Nonresponder)F_1 and Nonresponder B Cells

Splenic non-T cells in culture	(B6A)F_1 T cell priming	Antigens in culture	Bystander helper activity*		
			Whole culture	B10.A cells	(B6A) F_1 B cells
B10.A‡	(TG)-A--L	SRBC + (TG)-A--L§	5 ± 16		
(B6A)F_1‡	(TG)-A--L	SRBC + (TG)-A--L	3,380 ± 960		
B10.A + (B6A)F_1‖	(TG)-A--L	SRBC + (TG)-A--L	2,040 ± 730	450	1,590
B10.A‡	KLH	SRBC + KLH¶	3,880 ± 960		
(B6A)F_1‡	KLH	SRBC + KLH	15,130 ± 5,050		
B10.A + (B6A)F_1‖	KLH	SRBC + KLH	7,890 ± 3,390	3,950	3,950

* Anti-SRBC PFC/10^6 T cells/culture ± SE.
‡ 2×10^6/culture.
§ 100 μg/ml.
‖ 10^6 each/culture.
¶ 5 μg/ml.

TABLE VII

I Region Gene Expression in Bystander Helper T Cell Activity

Splenic non-T cells in culture	Peritoneal washings*	KLH-primed T cells	Antigens in culture	Bystander helper activity*
B10.A	—	(B6A)F_1	SRBC + KLH§	3,110 ± 460
B10	—	(B6A)F_1	SRBC + KLH	2,700 ± 370
B10.A	—	(B6A)F_1 → B10.A	SRBC + KLH	24,300 ± 2,470
B10.A	(B6A)F_1	(B6A)F_1 → B10.A	SRBC + KLH	24,260 ± 3,060
B10	—	(B6A)F_1 → B10.A	SRBC + KLH	140 ± 60
B10	(B6A)F_1	(B6A)F_1 → B10.A	SRBC + KLH	10 ± 440
B10.A	—	(B6A)F_1 → B10.A	SRBC	270 ± 50
B10.A	(B6A)F_1	(B6A)F_1 → B10.A	SRBC	750 ± 290
B10	—	(B6A)F_1 → B10.A	SRBC	30 ± 10
B10	(B6A)F_1	(B6A)F_1 → B10.A	SRBC	340 ± 110

* 10^5 cells from washings of normal mice/culture.
‡ Anti-SRBC PFC/10^6 T cells per culture ± SE.
§ 5 μg/ml.

help the anti-SRBC response of responder B cells and MØ, and not of nonresponder B cells and MØ in the presence of (TG)-A--L. Surprisingly, however, the *Ir* genes in this case were expressed at least by the B cell because, under most circumstances, nonresponder B cells could not be stimulated to respond to SRBC by this bystander mechanism, even if adequate responder or F_1 antigen presenting MØ were added to the cultures. Thus, nonresponder B cells failed to mount an anti-SRBC response in the presence of responder splenic non-T cells, (responder × nonresponder)F_1, irradiated splenic non-T cells, or (responder × nonresponder)F_1 peritoneal washings. These last two types of cells were shown to be adequate sources of antigen-presenting cells because they could be used to reconstitute an anti-SRBC response of responder Sephadex G-10-passed B cells driven by (TG)-A--L-primed T cells plus (TG)-A--L, or an anti-SRBC response of either responder or nonresponder Sephadex G-10-passed B cells driven by KLH-primed T cells plus KLH.

Only in two types of experiments did we observe a partial response to SRBC of B10.A, nonresponder B cells, driven by (TG)-A--L plus (TG)-A--L-primed T cells. Under both circumstances, the likelihood of potentially interactive T cells contaminating our B cell preparations had been increased. Thus, after Sephadex G-10 passage of B10.A B cells, these cells were then stimulated to mount a small response to SRBC in the presence of (TG)-A--L-primed T cells, (TG)-A--L, and (B6A)F_1-irradiated spleen cells (Table V). This small stimulation was not observed if the B10.A cells had not been Sephadex G-10 passed (Table III). In our hands, Sephadex G-10 passage yields about 25% of the starting population of cells and, therefore, in addition to removing MØ, certainly removes other populations of splenic cells, perhaps especially the more adherent B cells. Contaminating T cells may, therefore, be enriched by this procedure, as we are aware that even our most stringent anti-T plus C treatments do not entirely remove these cells (24). Such enriched contaminating cells might recognize SRBC or alloantigens and, in addition, be activated to release B cell stimulating factors by TCGF secreted by (TG)-A--L-primed T cells responding to (TG)-A--L.

The idea that it is contaminating T cells that cause the partial stimulation of B10.A

B cell responses to SRBC driven by (TG)-A--L-primed T cells plus (TG-A--L) is supported by the observation that nonirradiated (B6A)F_1, but not B10, splenic non-T cells stimulated similar small responses (Table VI). These small responses were not stimulated in the presence of irradiated (B6A)F_1 splenic non-T cells. Again, the possibility of activating by TCGF production, SRBC-specific contaminating T cells in these preparations existed, if they were not irradiated. In the case of (B6A)F_1, T cells, but not B10 T cells, these could then interact directly with the B10.A cells (12, 18). Our future experiments will investigate these points more thoroughly.

Thus, it appears that in the majority of experiments (TG)-A--L-specific, *Ir* gene expression by B cells at least governed the ability of these cells to respond in bystander experiments to SRBC driven by (TG)-A--L plus (TG)-A--L-primed T cells. Similar results were obtained when studying the *I*-region restriction of bystander helper T cells primed to KLH. Thus [(B6A)F_1 → B10.A] KLH-primed T cells were able to deliver KLH-specific bystander help only to B10.A cells that responded to SRBC, not to B10 B cells. This result was unaffected by providing the cultures with (B6A)F_1 accessory cell function, which suggested that *I*-region genes were functioning at least in the B cells in these cultures.

Although bystander helper T cells have been recognized for some years, their mode of action has not been clearly established previously. The most straightforward explanation has always been that the T cells interact with antigen on the surfaces of accessory cells, such as MØ, and that they there secrete nonspecific helper factors that diffuse through the culture medium and act on bystander B cells that have bound RBC. This simple explanation is supported by the observation that bystander help is relatively difficult to demonstrate in vivo, where one might expect such factors to be very short range (25, 26). The explanation is not, however, borne out by the results reported in this paper. Were it true, one would not expect the bystander helper T cells to be affected by *Ir* or *I*-region genes expressed by the B cells themselves, especially in the presence of adequate accessory cell function of the appropriate *H-2* type.

Recently, others have investigated the problem of B cell *I* region restriction of nonspecific help delivered by T cells that responded to irrelevant antigens. In one case Augustin and Continho (27) showed that T cells primed to minor antigens would only help B cell polyclonal responses if the T cells could interact directly with the relevant B cells. In two other cases (28, 29), direct interaction with B cells was not found to be necessary, in contradiction to the results reported in this paper. This difference will be discussed in greater detail below.

Two alternative explanations for bystander help may, therefore, be offered that suggest that, for effective help to be delivered, the bystander helper T cell and target B cell must come into direct contact. The first explanation suggests that this direct contact is mediated by recognition of antigen, (TG)-A--L or KLH in the experiments described in this paper, plus *I* or *Ir* gene products on the surface of the SRBC-binding B cell by helper T cells (Fig. 1 a). Such a theory accounts for the *I* and *Ir* gene functions expressed by B cells in the experiments described in this paper. It may also explain the high antigen concentration required in vitro for bystander helper to be manifested. We would suggest that only at such high antigen concentrations is enough (TG)-A--L or KLH bound nonspecifically to B cell surfaces to be recognized by helper T cells. The theory would predict that at high concentrations of these antigens, their

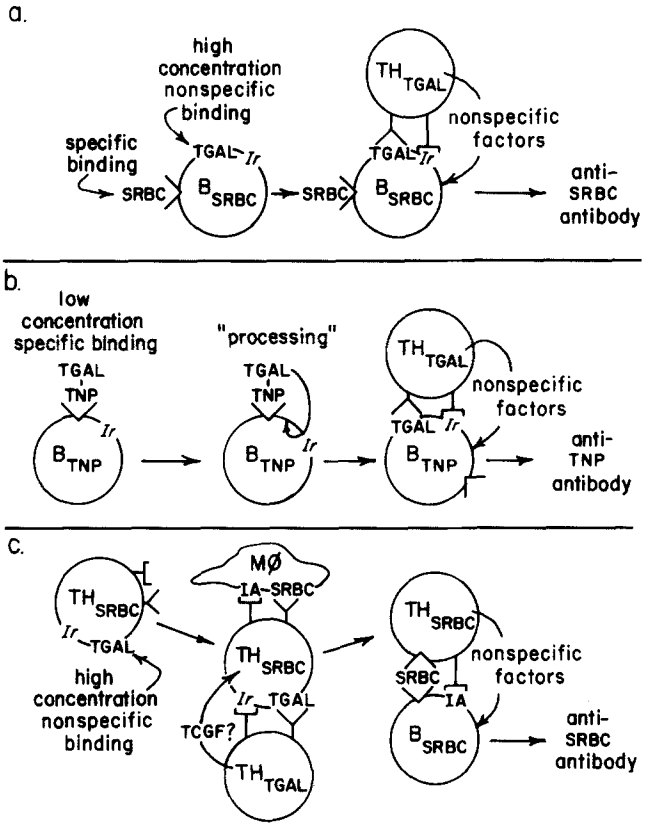

FIG. 1. Models of *Ir*-restricted interactions between helper T cells and B cells. a. Recognition of (TG)-A--L nonspecifically bound to SRBC-specific B cells may mediate (TG)-A--L-driven bystander help. b. Model showing how recognition of antigen on B cell surface after binding through the immunoglobulin receptor may mediate antigen-specific help. c. Model showing how recognition of (TG)-A--L nonspecifically bound to SRBC-specific T cell helper precursors may mediate (TG)-A--L-driven bystander help. See Discussion for discussion of this figure. TH, helper T cell.

nonspecific binding to B cell surfaces should be observable. This is in fact the case; we have already shown that high concentration causes fluoresceinated bovine serum albumin to be bound to 6% or more of splenic B cells (30). Moreover, preliminary experiments have demonstrated that 1 mg/ml fluoresceinated KLH binds to up to 50% of splenic B cells, and 1 mg/ml fluoresceinated (TG)-A--L to 11% of the same cell type (Personal observation.), as detected by direct immunofluorescence.

One attractive feature of this theory is that it allows for B cell presentation of antigen to T cells by a mechanism in which the antigen is not bound to the B cell immunoglobulin receptor. Because helper T cells are primed by, and recognize, antigen plus *I*-region products bound to MØ surfaces (31–33) in the apparent absence of immunoglobulin receptors, it has always been a paradox that they are then able to interact with B cell-bound antigen and *I*-region products (12, 15, 18, 34–36) where, it was presumed, the antigen was bound via immunoglobulin. Such a paradox could be resolved by assuming two receptors, one for antigen and one for *I* region product for the helper T cell. This resolution still did not explain the specificity of helper T cells

for determinants on what was presumed to be processed antigen on MØ surfaces, but native antigen in the B cell immunoglobulin receptor. One could resolve this problem by suggesting that helper T cells recognize only processed antigen, and that this processing can be mediated either by MØ or B cells. For the latter cell type, however, high antigen concentrations on the B cell surface would be required to allow processing to occur. This could be achieved either by focusing the antigen on a particular B cell by means of the B cell's immunoglobulin receptor (Fig. 1 b), or by raising the antigen concentration in the milieu to abnormally high levels. Theories and explanations of this type have been put forward in the past (37, 38).

Therefore, the theory outlined above predicts that, under condition of limiting bystander help, nonspecific helper molecules are most efficiently delivered to the B cell by T cells that interact directly with *Ir* gene or *I-A* products plus antigen on the surface of the responding B cell itself. Such a prediction does not preclude the idea, however, that nonspecific helper molecules may also be secreted by T cells that interact with antigen plus *I*-region products on other B cells, or on MØ. Such molecules might then diffuse to potentially SRBC-reactive B cells and stimulate them. One would only expect this mechanism to operate, however, under conditions where helper T cells were less limiting. This may be the explanation for the lack of B cell restriction in bystander help experiments reported by Hunig and Schimpl (28) and Schreier et al. (29).

A second, though less likely, explanation for the results presented in this paper is that bystander helper T cells act by recognizing antigen on some accessory cell, and *Ir* and *I*-region gene products on the B cell simultaneously. Direct contact with the B cell would, therefore, be achieved by *Ir* or *I* recognition only on the B cell surface. This explanation would not agree with observations made for cytotoxic cells (39).

Several theories are consistent with the results outlined in this paper, and do not include direct T cell-B cell contact in their explanation. For example, factors such as TCGF have recently been shown to be remarkable inducers of T cell activity, in the presence of antigen and in cultures of cells that were apparently T cell depleted (40). One could, therefore, hypothesize that the mechanism of bystander help involved the induction of SRBC-specific helper T cells that contaminated our B cell preparations by F_1 T cells responding to (TG)-A--L or KLH (Fig. 1 c). We feel that this is not a very likely explanation for our results, as bystander help for $H\text{-}2^a$ strains responding to (TG)-A--L is apparently governed by a B cell-expressed *Ir* gene, and we have previously shown that this *Ir* gene may not be expressed in $H\text{-}2^a$ helper T cells (12). Our future experiments will be designed to test this last theory more specifically, however, and also to investigate the role of accessory cells in bystander help.

Summary

The mode of action by bystander helper T cells was investigated by priming (responder × nonresponder) (B6A)F_1 T cells with poly-L-(Tyr, Glu)-poly-D,L-Ala--poly-L-Lys [(TG)-A--L] and titrating the ability of these cells to stimulate an anti-sheep red blood cell (SRBC) response of parental B cells and macrophages in the presence of (TG)-A--L. Under limiting T cell conditions, and in the presence of (TG)-A--L, (TG)-A--L-responsive T cells were able to drive anti-SRBC responses of high-responder C57BL/10.SgSn (B10) B cells and macrophages (MØ), but not of low-responder (B10.A) B cells and MØ. Surprisingly, the (TG)-A--L-driven a nti-SRBC

response of B10.A B cells was not restored by addition of high-responder accessory cells, in the form of (B6A)F_1 peritoneal or irradiated T cell-depleted spleen cells, or in the form of B10 nonirradiated T cell-depleted spleen cells. These results suggested that (TG)-A--L-specific *Ir* genes expressed by B cells controlled the ability of these cells to be induced to respond to SRBC by (TG)-A--L-responding T cells, implying that direct contact between the SRBC-binding B cell precursor and the (TG)-A--L-responsive helper T cells was required. Analogous results were obtained for keyhole limpet hemocyanin (KLH)-driven bystander help using KLH-primed F_1 T cells restricted to interact with cells of only one of the parental haplotypes by maturing them in parental bone marrow chimeras.

It was hypothesized that bystander help was mediated by nonspecific uptake of antigen [(TG)-A--L or KLH] by SRBC-specific B cells and subsequent display of the antigen on the B cell surface in association with *Ir* or *I*-region gene products, in a fashion similar to the MØ, where it was then recognized by helper T cells. Such an explanation was supported by the observation that high concentrations of antigen were required to elicit bystander help. This hypothesis raises the possibility of B cell processing of antigen bound to its immunoglobulin receptor and subsequent presentation of antigen to helper T cells.

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