T CELLS COOPERATING IN THE INDUCTION OF DELAYED-TYPE HYPERSENSITIVITY ACT VIA THE LINKED RECOGNITION OF ANTIGENIC DETERMINANTS*

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A requirement for specific cellular collaboration has been demonstrated for the induction of humoral antibody (1-3), cytotoxic T cells (4, 5), and for those helper T cells able to help the induction of B cells (6). Leung and Ada (7) have recently shown that antigen-specific cells can significantly enhance the primary in vitro induction of delayed-type hypersensitivity (DTH)[1] to influenza virus. We independently developed an in vitro system in which the induction of significant DTH reactivity is dependent on the presence of antigen-specific helper T cells. We used this system to determine the antigen recognition requirements by which these helper T cells act in the induction of DTH precursor cells.

A phenomenon referred to as carrier specificity in the elicitation of DTH has been reported in the literature (8, 9), i.e., animals sensitized to a hapten on a given carrier will respond with DTH reactivity only when challenged with the hapten on the same carrier as was used for sensitization. This phenomenon has been insufficiently characterized to ascertain whether it is simply quantitative, in that only a small amount of the DTH reactivity is directed against the hapten, or whether it reflects either a requirement for specific cellular collaboration in the elicitation of DTH and/or that the DTH effector cells are specific for neoantigenic determinants created by coupling the hapten to the carrier. In any case, the phenomenon does not bear on the cellular requirements for inducing DTH and is in this sense different from the classical carrier effects described for the induction of humoral responses.

The mechanism by which antigen-specific cells cooperate in the induction of B and helper T cells is known to be most effective when the two specific classes of cells recognize determinants that are physically linked to one another (10, 11). With the recent establishment of experimental systems that allow DTH to be induced in vitro (12, 13), it has become possible to determine the cellular requirements for the induction of DTH. The experiments described in this paper were designed to test whether (*a*) specific cellular collaboration was required for the induction of DTH and

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[1] *Abbreviations used in this paper:* BRBC, burro erythrocytes; CRBC, chicken erythrocytes; DTH, delayed-type hypersensitivity; FCS, fetal calf serum; FGG, fowl gamma globulin; FGG-BRBC, fowl gamma globulin physically coupled to burro erythrocytes; FGG-MRBC, fowl gamma globulin physically coupled to mouse erythrocytes; i.v., intravenous; MRBC, mouse erythrocytes; RBC, erythrocytes; s.c., subcutaneous; Thy-1, thymus-derived cell marker.

(*b*) whether the putative helper cells must recognize determinants that are physically linked to the antigen against which DTH is induced to cooperate effectively.

Materials and Methods

Mice. CBA/CaJ mice, aged 6–12 wk, were obtained from the University of Alberta animal facility. Male or female mice were used, the sex being kept constant within individual experiments.

AKR/J mice, aged 6–12 wk, were obtained from The Jackson Laboratory, Bar Harbor, ME.

Antigens. Burro erythrocytes (BRBC) were purchased in Alsever's solution (Colorado Serum Co., Denver, CO). Heparinized chicken erythrocytes (CRBC) were collected from a particular bird kept at the University of Alberta animal facility and were washed and stored refrigerated in Leibovitz medium. Mouse erythrocytes (MRBC) were collected in Alsever's solution from CBA/CaJ mice. All RBC were washed three times with sterile saline before use in culture.

Fowl gamma globulin (FGG) anti-BRBC and FGG anti-MRBC-specific antisera were made by injecting chickens with appropriate RBC using the method of Miller and Warner (14). The antiserum with anti-MRBC activity was actually raised against rat RBC but cross-reacted strongly with MRBC and not at all with BRBC. The antisera were titered by hemagglutination, and a twofold lower amount than that required for agglutination was used to couple antibody to RBC for use in culture. The antiserum and RBC were incubated in a 37°C waterbath for 30–60 min and washed three times with physiological saline before use in culture. The coupled cells were also titered by hemagglutination with a mouse anti-FGG antiserum to ensure that the coupling procedure had been successful.

Immunization Procedure. 5×10^8 RBC in a volume of 50 μl were injected subcutaneously into the hind footpads of mice 6 d before killing, to obtain helper cells for DTH to RBC antigens. 0.5 mg of purified, lyophilized FGG (12) was dissolved per 0.2 ml of physiological saline and injected intravenously 2 or 4 d before killing to obtain FGG-specific helper cells. Spleen cells were always used as the source of helper cells from these animals.

Culture System. Single spleen cell suspensions were prepared in Leibovitz medium by mincing the spleens and gently passing the cells through a stainless steel wire mesh. After the cell clumps had settled for 2 min on ice, the cells suspended in the supernatant were removed, washed twice in Leibovitz medium, and the viable white cells were counted by trypan blue exclusion. Those cells requiring irradiation were exposed as a single cell suspension to a ^{137}Cs source (Gamma Cell 40, Atomic Energy of Canada Ltd., Ottawa, Canada) for the time required to receive 1,500 rad (~15.9 min). The cells were again washed before being added to the cultures.

The culture medium consisted of Eagle's minimum essential medium supplemented with 10% fetal calf serum (FCS) (Gibco, Grand Island Biological Co., Grand Island, NY), 10^{-4} M 2-mercaptoethanol, and 50 μg/ml gentamicin (Shering Co., Kenilworth, NJ). Each Costar well contained 4 ml of culture medium, 10^7 viable responder white cells, and, where required, 10^7 irradiated viable white cells and 10^7 RBC as antigen where appropriate. The cultures were maintained at 37°C in an atmosphere of 10% CO_2 for 6 d.

Assay of the DTH Response. The viable cells (30–50% of input number) were collected on Isopaque-Ficoll gradients (15), washed, and injected subcutaneously at a dose of 10^7 cells per hind footpad with or without 5×10^7 RBC, and the footpad swelling was measured in units of 10^{-2} mm after 24 h (12). Results recorded in this paper are expressed as antigen-specific swelling, i.e., the mean swelling of five mice given 10^7 sensitized cells plus antigen minus the mean swelling of five mice given 10^7 sensitized cells without antigen. This latter swelling never exceeded 20 U in these experiments. The SE is calculated as the square root of the sum of the squares of the SE of the swelling observed with and without antigen.

Preparation and Use of Anti-Thy-1 Antisera. AKR anti-CBA Thy-1 antiserum was prepared by a method developed by I. A. Ramshaw (personal communication). AKR mice were injected intravenously with 5×10^7 CBA thymocytes, and the serum collected after 7 d was specific for CBA T cells, having no detectable activity against AKR T cells. Anti-Thy-1 treatment of cells consisted of incubating 2×10^7 cells/ml in a 1:5 dilution of anti-Thy-1 antibody on ice for 20 min, washing, and resuspending the cells in 1 ml of a 1:5 dilution of rabbit complement. After 45 min in a 37°C waterbath, the cells were washed, and the number of viable cells was

estimated by trypan blue exclusion. A more efficient commercial monoclonal anti-Thy-1.2 IgM antibody (New England Nuclear, Lachine, Quebec) was also available. In this case, 10^7 cells per ml were incubated on ice in a 1:10,000 dilution of the monoclonal antibody for 60 min, and agarose-absorbed guinea pig complement (16) was added to a final dilution of 1:16. The remainder of the procedure was identical to that described above for the conventional antibody preparation. Cells were also incubated under identical conditions, except that the specific antibody was not added to ensure that any loss of activity of the treated cells required the presence of anti-Thy-1 antibody.

Results

Conditions for the Induction of DTH In Vitro. A variety of experimental conditions have been used in this laboratory to induce DTH reactivity in vitro, including Diener-Marbrook cultures, Costar dishes (35 mm), Linbro trays (15 mm), and microtiter trays (6 mm). The most critical factors determining whether DTH or some other class of immunity is induced are the densities of the responding cells and the concentration of antigen in culture. The large Costar dishes were chosen for these experiments because the number of cells required after 6 d of culture is of the order of 10^8 (10^7 per mouse, five mice ± antigen). These dishes allowed maximum induction when they contained a volume of 4 ml of medium. Fig. 1 shows the antigen-specific swelling induced with various cell numbers and antigen concentrations after 6 d of culture in Costar dishes. A high antigen concentration (10^9 RBC/well) allows the induction of DTH in cultures containing a minimum of 10^7 normal spleen cells but not in cultures containing one-half that number. DTH is effectively induced at doses of 1×10^7 to 2×10^7 normal spleen cells per well when 10^9 BRBC are provided as antigen but not with one one-hundredth of that concentration. 10^7 BRBC provide sufficient antigen to induce DTH in Costar dishes that contain 3×10^7 to 3.5×10^7 normal spleen cells per well (data not shown), but these dense cultures require a change of medium during the incubation to ensure a reasonable yield of viable cells.

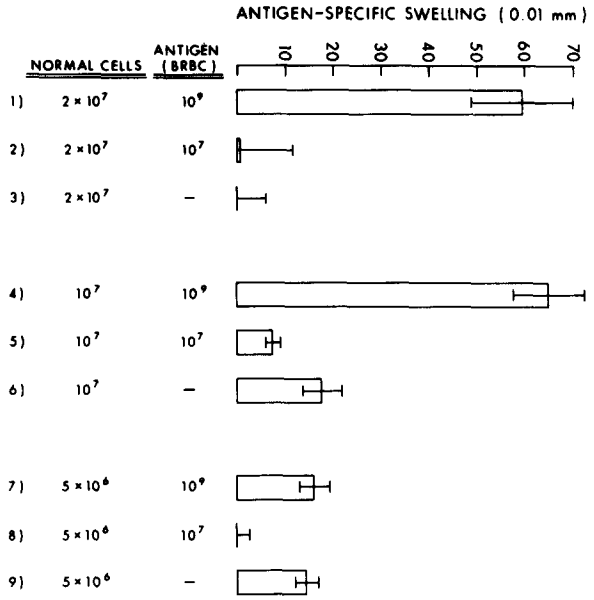

FIG. 1. Dependence of in vitro induction of DTH on cell density and antigen concentration.

These observations are understandable if cellular collaboration between DTH precursor cells and helper T cells is essential for the induction of DTH, and the cooperative event becomes more infrequent as the density of normal spleen cells is decreased, especially at the lower antigen concentrations. This working hypothesis is testable by choosing those culture conditions where the induction of DTH is undetectable and providing antigen-primed cells as a putative source of help; if the hypothesis is correct, such cells should facilitate the induction of DTH. The source of help must be such that it will contribute to the antigen-specific induction of immune reactivity but will not contribute functional precursor cells to the cultures for one to be able to infer that cellular cooperation is taking place. Irradiated spleen cells from antigen-primed animals have been used in the past to provide such a source of helper activity for the humoral response (17) or the cytotoxic T cell response (5). Irradiation does not allow the cells to divide and differentiate, but cells that are fully differentiated at the time of the irradiation and do not have to divide to express reactivity can apparently function until their death (~48 h under these culture conditions). Primed irradiated spleen cells were tested in this experimental system to see whether the addition of helper cells might allow the induction of DTH in those cultures where it seemed likely that the amount of help was limiting the induction of DTH.

A Thy-1-bearing Cell Acts as a Helper Cell in the Induction of DTH In Vitro. Attempts were made to immunize mice with BRBC in such a way that they would provide the putative helper cells for the induction of DTH in those cultures containing 10^7 normal spleen cells and 10^7 BRBC. Any cells added to this basic system were irradiated with 1,500 rad before being added to the cultures. The addition of irradiated normal spleen cells (10^7, 2×10^7, or 4×10^7) did not result in the induction of DTH. Spleen cells from mice immunized with large doses (10^9) of BRBC intravenously that mount a humoral response gave insignificant help to the cultures over a large range of doses (10^4 to 2×10^7) and at periods up to 1 wk after immunization, even after these cells were passed over a nylon wool column to remove antibody-producing and B cells. Mice given low doses (2×10^5, 10^6) of BRBC intravenously did contribute some helper activity when their spleen cells were used 2, 3, or 4 d after immunization (Table I). Titration of these primed irradiated cells showed that the helper activity peaked at a dose of 10^7 irradiated cells per Costar well. Spleen cells from mice given 5×10^8 BRBC s.c. in the hind footpads 6 d previously consistently contained helper activity (Table I), with maximum activity again occurring in the presence of 10^7 irradiated primed cells per well. Mice primed with CRBC intravenously or subcutaneously contributed maximum helper activity with similar doses and kinetics.

Spleens from mice primed 6 d previously with 5×10^8 RBC s.c. were chosen as the helper population and routinely used. It can be seen from Fig. 2 that DTH is only induced in those cultures that contain primed irradiated spleen cells as opposed to normal irradiated spleen cells (rows 2 vs. 1; 7 vs. 6). Treatment of the helper population with anti-Thy-1 antiserum and complement before addition to the cultures results in a loss of the induction of DTH (rows 2 and 4), although treatment with complement alone does not significantly reduce the amount of DTH induced (rows 2 and 3; 7 and 8). Those primed cells that have been treated with anti-Thy-1 antiserum and complement do not provide help when supplemented with normal irradiated spleen filler cells (row 5). This indicates that the anti-Thy-1 antibody treatment is removing a T cell from the primed population that is not present in great

TABLE I

Effect of Route and Kinetics of Immunization on Generation of Radioresistant Helper Cell Activity by Induction of RBC-specific DTH in Culture

Antigen*		Route‡	Days§	Antigen-specific swelling (10^{-2} mm)‖
BRBC	CRBC			
—	—	—	—	1 (10)
10^6	—	i.v.	2	22 (13)
10^6	—	i.v.	3	30 (5)
10^6	—	i.v.	4	45 (1)
—	—	—	—	5 (2)
5×10^8	—	s.c.	2	15 (2)
5×10^8	—	s.c.	4	30 (3)
5×10^8	—	s.c.	6	50 (3)
—	—	—	—	14 (3)
—	5×10^8	s.c.	2	13 (2)
—	5×10^8	s.c.	4	12 (9)
	5×10^8	s.c.	6	59 (4)

* Antigen refers to the dose of erythrocytes used to prime the mice whose spleen cells provided a source of helper cells for these experiments.

‡ Route refers to the route of immunization of those mice providing helper cells.

§ Days refers to the length of time between immunization of the mice and collection of the spleen cells for the helper experiments.

‖ Antigen-specific swelling is calculated as the mean swelling (in 10^{-2}-mm units) of five mice given 10^7 sensitized cells and 5×10^7 RBC minus the mean swelling of five mice given 10^7 sensitized cells without antigen. The SE of the difference is represented by the numbers in parentheses and is calculated as the square root of the sum of the squares of the SE with and without antigen.

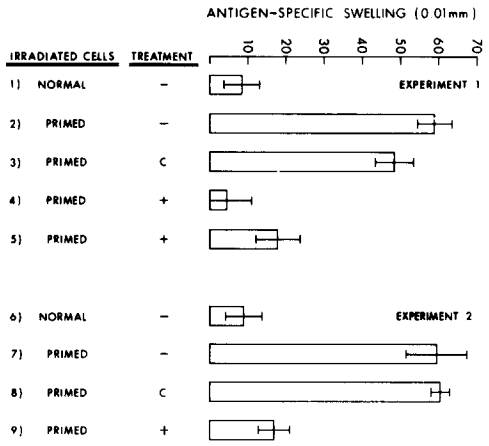

FIG. 2. A Thy-1-bearing cell acts as a helper cell in the induction of DTH. Irradiated cells received 1,500 rad of gamma irradiation before being added to the cultures. These came from unprimed mice (normal) or mice primed 6 d previously with 5×10^8 BRBC subcutaneously in the footpad. The population of helper cells was treated before being added to the cultures with anti-Thy-1.2 antibody and complement (+) (yield ~70%) or with complement alone (C') (yield ~83%). Row 5 is equivalent to row 4 except that normal irradiated spleen filler cells have been added such that 10^7 cells were added per culture.

numbers in normal spleen and is consistent with the Thy-1-bearing cell being antigen specific.

The DTH Effector Cells Are Derived from the Normal Spleen Cell Population. The possibility existed that the DTH activity observed was actually the result of some cells derived from the primed spleen cell population, even though this would require them to have survived 6 d in culture after receiving 1,500 rad of irradiation. The Thy-1 cell marker was used to determine which population of cells gave rise to the DTH effector cells. Spleen cells were harvested from two mouse strains that carry the same major histocompatibility antigens but differ in their Thy-1 alleles, i.e., CBA/CaJ and AKR/J. Mice of one strain were primed, their cells irradiated, and used to cooperate in culture with normal spleen cells of the same or the other mouse strain. At the end of the incubation, the DTH effector cells were treated with antiserum specific for Thy-1.2, i.e., the CBA/CaJ antigen.

As seen in Fig. 3, the antiserum and complement treatment completely eliminated any footpad swelling in those mice given sensitized cells from cultures in which CBA/CaJ normal spleen cells had been incubated with AKR/J irradiated primed spleen cells (rows 6 and 7) but not in those mice given cells from cultures in which AKR/J normal spleen cells had been incubated with CBA/CaJ irradiated primed spleen cells (rows 2 and 3). This shows that the DTH effector cells are derived from the normal spleen cell population and not from the primed irradiated spleen cells used as a source of helper activity in these experiments. They also demonstrate that the cells that mediate the DTH swelling reaction are indeed T cells, confirming other observations in which the DTH effector cells, obtained with syngeneic helper and

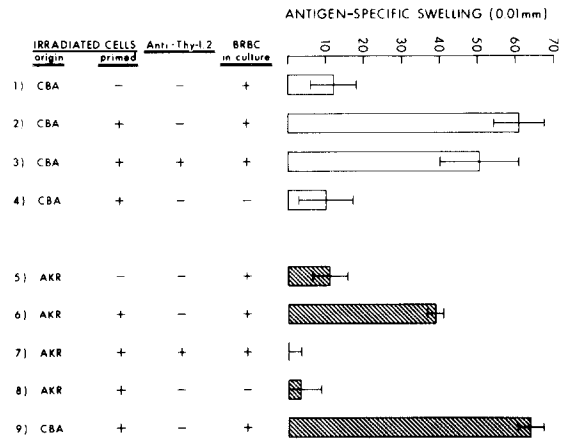

FIG. 3. The DTH effector cells are derived from the normal spleen cell population. Irradiated cells received 1,500 rad of gamma irradiation before being added to the cultures. These came from unprimed mice (–) or mice primed 6 d previously with 5×10^8 BRBC s.c. in the footpad (+). These cells were from either CBA/CaJ or AKR/J strains of mice and were added to cultures containing normal spleen cells from either CBA/CaJ (hatched bars) or AKR/J (open bars). The DTH effector cells generated after 6 d in culture were treated with anti-Thy-1.2 antiserum (specific for the CBA/CaJ antigen) and complement before being injected into the footpads of normal CBA/CaJ mice. This treatment killed 30% of the viable cells after normal spleen cells from AKR/J mice had been cultured with primed irradiated cells from CBA/CaJ mice and 82.5% after normal spleen cells from CBA/CaJ mice had been cultured with primed irradiated cells from AKR/J mice. These cells were supplemented with normal spleen cells from CBA/CaJ mice such that 10^7 cells were injected per footpad.

precursor cells, were shown to be Thy-1 positive (Tucker and Bretscher, unpublished observations).

Control experiments show that allogeneic effects resulting from minor histocompatibility differences are not responsible for the helper activity of the irradiated population. Thus, when normal cells of one strain are incubated with irradiated normal cells of the other strain in the presence of antigen, no significant antigen-specific swelling is induced (rows 1 and 5); in contrast, when irradiated primed cells of one strain are incubated with the normal cells of the other strain in the presence of antigen, significant DTH is induced (rows 2 and 6). Furthermore, the induction of DTH in this nonsyngeneic situation still requires the presence of antigen (rows 4 and 8). Finally, it can be seen from Fig. 3 that the DTH induced in cultures containing responder cells of either strain is not significantly different when each is helped by CBA/CaJ-primed irradiated spleen cells (rows 2 and 9). In conclusion, these experiments show that the DTH effector cells are derived from the normal spleen cell population.

The Helper T Cell for the Induction of DTH Is Antigen Specific. Mice were primed with either BRBC or CRBC and their irradiated spleen cells used as source of helper cells to induce DTH effector cells by culturing normal spleen cells with either the same or the other erythrocyte antigen. From the results shown in Table II, it can be seen that

TABLE II

Helper T Cell Is Antigen Specific

Priming*	Antigen culture‡	Footpad§	Antigen-specific swelling (10^{-2} mm)‖	
			Experiment 1	Experiment 2
—	BRBC	BRBC	5 (5)	2 (2)
BRBC	BRBC	BRBC	81 (6)	68 (8)
BRBC	CRBC	BRBC	2 (1)	8 (6)
BRBC	CRBC	CRBC	10 (6)	6 (6)
—	CRBC	CRBC	6 (5)	4 (2)
CRBC	CRBC	CRBC	68 (7)	45 (8)
CRBC	CRBC	BRBC	6 (2)	ND¶
CRBC	BRBC	CRBC	−9 (7)	10 (3)
CRBC	BRBC	BRBC	0 (1)	11 (3)
BRBC	BRBC + CRBC	BRBC	72 (7)	ND
BRBC	BRBC + CRBC	CRBC	12 (7)	ND

* Animals were primed subcutaneously in the footpad with 5×10^8 of the appropriate RBC 6 d before their spleens were removed and used as a source of helper cells.

‡ Cultures contained 10^7 normal spleen cells and 10^7 of the designated RBC as antigen.

§ 10^7 viable harvested cells were injected per footpad with 5×10^7 of the designated RBC as antigen.

‖ Antigen-specific swelling is calculated as the mean swelling (in 10^{-2} mm units) of five mice given 10^7 sensitized cells and 5×10^7 RBC minus the mean swelling of five mice given 10^7 sensitized cells without antigen. The SE of the difference is represented by the numbers in parentheses and is calculated as the square root of the sum of the squares of the SE with and without antigen.

¶ Not done in this particular experiment.

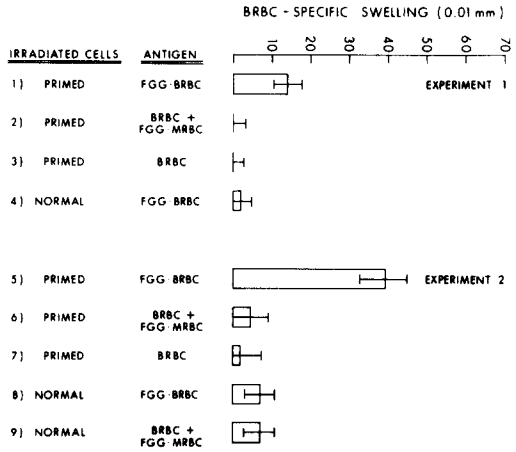

FIG. 4. Irradiated helper T cells for the induction of DTH act via linked recognition. Irradiated cells received 1,500 rad of gamma irradiation before being added to the cultures. These came from unprimed mice or mice primed intravenously 2 d (experiment 1) or 4 d (experiment 2) previously with 0.5 mg of soluble FGG in saline. The conjugated antigens were prepared by incubating specific fowl-anti-RBC antibody at a twofold lower than hemagglutinating concentration for 30–60 min at 37°C. BRBC are present in all cultures, either linked to FGG or not, and the DTH is measured against BRBC.

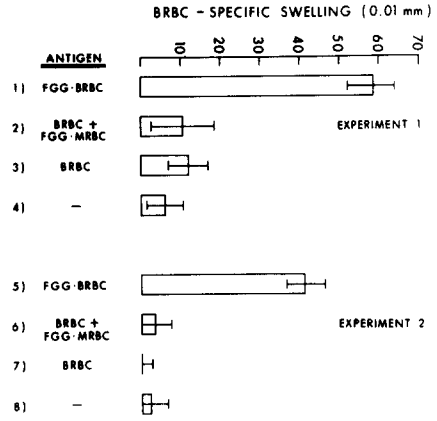

FIG. 5. FGG-specific helper cells for the induction of BRBC-specific DTH act via linked recognition. These FGG-specific cells are the same cells used in the experiments reported in Fig. 4, except that these are unirradiated and providing a source of BRBC-specific precursor cells as opposed to helping induce a population of normal spleen cells. The FGG-specific helper cells are from the spleens of mice primed intravenously 2 d (experiment 1) or 4 d (experiment 2) previously with 0.5 mg of soluble FGG in saline.

swelling activity is induced and elicited only when the same erythrocyte antigen is present during the in vivo priming, the in vitro induction period, and elicitation. This demonstrates a lack of cross-reactivity between these two erythrocyte antigens at the level of the helper population and the DTH effector population and shows that the help is acting in an antigen-specific fashion. Thus, the spleen cells from mice primed with BRBC only provide helper activity for the induction of BRBC-specific DTH and only when BRBC are present in the culture system. Cells from mice primed with CRBC only help induce CRBC-specific DTH and only when CRBC are present in

the culture, and this can only be detected when CRBC are also present in the footpad during elicitation. It can also be seen from Table II that the presence of BRBC-primed spleen cells and both erythrocyte antigens in culture only results in the induction of BRBC-specific DTH. These results argue against the possibility that a nonantigen-specific long-range factor(s) released as a consequence of an interaction between the helper T cell and antigen allows DTH precursor cells to be induced.

The Helper T Cell for the Induction of DTH Acts via Linked Recognition. To find out whether the helper cell for the induction of DTH acts via linked recognition, it was necessary to have two noncross-reacting antigens that could be given either separately or as a conjugate. If helper T cells act via linked recognition, helper cells specific for one antigen would allow the induction of DTH specific for the other antigen only when the conjugate was present in the cultures. FGG-specific helper cells were obtained from the spleens of mice injected with 0.5 mg of FGG i.v. 2 or 4 d previously. These cells were irradiated and tested to see whether they would cooperate with normal spleen cells to induce BRBC-specific DTH in cultures containing either the conjugate FGG-BRBC or BRBC plus the conjugate FGG-MRBC. The results shown in Fig. 4 demonstrate that BRBC-specific DTH is induced only when FGG-BRBC is provided as the antigen in cultures containing FGG-specific helper cells (rows 5 and 1). Although the helper cells obtained after 2 d of in vivo priming with FGG allow the induction of a small antigen-specific swelling (significantly different from the control groups, i.e., rows 1 vs. 2, 3, 4; $P < 0.005$ by Student's t test), the cells obtained after 4 d of priming provide sufficient help to induce a good DTH response in those cultures that contain the FGG-BRBC conjugate as antigen. BRBC-specific DTH is not induced when both BRBC and FGG (linked to MRBC) are present in the cultures, i.e., physical linkage between the antigen against which the helper T cells are raised and the antigen inducing the DTH precursor cell is required (rows 5 vs. 6, 1 vs. 2). As can be seen from rows 5 vs. 8, the conjugate requires the presence of FGG-primed cells to induce DTH to BRBC.

In these experiments the mice are primed with one antigen, and the DTH is induced against another non-cross-reacting antigen. It was not necessary to irradiate the helper population, as the FGG-primed cells did not given rise to BRBC-specific DTH when incubated with BRBC. The FGG-primed cell population could be used as the source of BRBC-specific DTH precursor cells. When these cells are incubated at 10^7 cells per well with 10^7 RBC, reliable BRBC-specific DTH was induced only in those cultures that contained the FGG-BRBC conjugate as antigen (Fig. 5). The nonirradiated cells from mice primed 2 d previously with FGG are seen to provide very effective help for the induction of BRBC-specific DTH, even though they did not provide very efficient help when irradiated. The nonirradiated cells from mice primed 4 d previously with FGG induce BRBC-specific DTH in cultures not significantly different from that induced in normal spleen cells that are complemented with irradiated FGG-specific helpers. These observations show that the FGG-specific help obtained after 2 d of in vivo priming is relatively sensitive to irradiation compared with the help obtained after 4 d of priming and is expected if the 2-d as opposed to the 4-d primed cells have to divide or further differentiate to provide adequate helper function.

Discussion

The dependency of the induction of DTH on the density of spleen cells and concentration of antigen present in culture has been carefully examined. A limiting

system was chosen in which the number of normal spleen cells and antigen could not by themselves induce DTH, but in which a sufficient number of precursor cells was known to be present such that, if induced, they would give rise to measurable DTH effector activity. The induction of antigen-specific DTH occurs in these limiting cultures when they are supplemented with antigen-specific, Thy-1 bearing, irradiated, primed spleen cells. These helper T cells have been detected in mice primed in a manner that induces DTH to the relevant antigen in the whole animal. It was not possible to consistently demonstrate help for a DTH response by T cells derived from a mouse mounting a humoral response. This might be because the two helper cells are different or because another regulatory cell, present under conditions that lead to a humoral response, inhibits the induction of DTH (18). It appears that the question of whether the same T cell can help in the induction of all classes of immune response can most clearly be resolved by studies with cloned, stable, helper T cell lines or biochemically purified, antigen-specific helper factors, as opposed to populations of primed spleen cells.

The studies reported here have clearly shown that the DTH effector cell is derived from the normal spleen cell population and not from the irradiated primed cells. It has also been shown that the helper T cell acts in an antigen-specific fashion. Those cultures containing both BRBC and CRBC with T helpers from mice primed with BRBC do not induce any DTH specific for CRBC. This observation shows that the helper T cells do not act in vitro by releasing a nonspecific, long-range factor. This is in contrast to other observations (19) that show that some helper T cells specific for an antigen A can help antihapten B cells in the presence of A and the hapten conjugated to an unrelated carrier. The fact that DTH reactivity against a second antigen (BRBC) can be induced in our system, but only when this second antigen is physically linked to the antigen with which the helper T cells were primed (FGG), argues against the helper T cells examined here having anti-idiotype specificity. Evidence in favor of anti-idiotype-specific helper T cells for the induction of B cells has been presented by others (20, 21).

We have not examined the question of whether there is any MHC restriction between DTH precursor cells and the helper T cells defined here, though some observations suggest that such a restriction exists (7). This question can only be approached satisfactorily by using cells from mutually tolerant donors and will be the subject of further investigation.

These studies using FGG-primed helper cells show that triple complexes of antigen, antigen-specific helper T cell (or helper T cell factor), and the antigen-specific precursor cell must be formed to induce DTH reactivity. This requirement provides an explanation for the observations described in Fig. 1 on the effect of varying responder spleen cell density and antigen dose on the induction of DTH. Consider the situation in which DTH is induced with 10^7 spleen cells and 10^9 BRBC. Sufficient triple complexes must be formed in such cultures for DTH precursor cells to be induced and their progeny detected by their swelling activity. It is to be expected that the efficiency of triple complex formation could be reduced either by decreasing the antigen concentration or decreasing the spleen cell density. In fact, when either the antigen concentration is reduced from 10^9 to 10^7 BRBC or the spleen cell density is dropped from 10^7 to 5×10^6 cells per well, DTH is no longer induced (see Fig. 1). In the former cultures, DTH is induced when the cells are complemented with irradiated

specific helper T cells, and in the latter situation the addition of antigen-primed irradiated spleen cells, presumably providing specific helper T cells, also allows DTH to be induced (Tucker and Bretscher, unpublished observations). It should be emphasized that as the yield of cells in these different cultures is approximately the same and as 10^7 viable cultured cells are used to determine the antigen-specific swelling, the activity measured is derived from the same number of precursors present at the initiation of culture. The relative swelling observed is then a measure of the relative efficiency of DTH precursor cell induction in these different cultures. These findings, together with those showing that helper T cells act by linked recognition, provide a reasonable semiquantitative basis for understanding how the amount of antigen and density at which spleen cells are cultured, and in particular their interdependence, affects the induction of delayed-type hypersensitivity.

Helper T cells can be induced in vivo because primed, as opposed to normal, spleen cells can provide specific helper function. It seems likely that helper T cells for the induction of DTH are also generated under those conditions of in vitro culture that lead to the production of DTH reactivity. These considerations stress that the helper T cells characterized here might act indirectly in aiding the production of DTH precursor cells by, for example, aiding the induction of more helper T cells. Our observations do not bear on whether one or more sequential cellular interactions occur in these cultures; however, if several occur, they all must act by the linked recognition of antigenic determinants, as one interaction mediated by a long-range nonspecific factor would obviate the need for the linked recognition observed in these studies.

The requirement for helper T cells in the induction of DTH has some important biological consequences. It may be, in analogy with B cells, that unstimulated precursor DTH cells binding antigen are inactivated (22, 23), whereas in the presence of helper T cells they are induced. This would provide a means of eliminating anti-self DTH precursors when an insignificant number of helper T cells specific for self antigens is normally present. The requirement for helper T cells in the induction of B cells has provided an understanding of how humoral autoimmunity to a self component can be induced by an antigen that cross-reacts with the self antigen. Helper T cells specific for the foreign epitopes can help in the induction of B cells specific for the cross-reacting epitopes in the absence of helper T cells specific for the self antigen. It seems likely, in view of the requirement for helper T cells in the induction of DTH demonstrated here, that stimulation by an antigen that cross-reacts with a self antigen and that induces DTH may cause the induction of cell-mediated autoimmunity.

Summary

Culture conditions have been established that allow the induction of delayed-type hypersensitivity (DTH) precursor cells present in a cell population derived from unsensitized spleen cells only when antigen-specific, radioresistant, Thy-1-bearing helper cells are added. This specific cellular cooperation acts via the linked recognition of two determinants on the antigen; thus, cells primed to the protein antigen fowl gamma globulin (FGG) will only allow the induction of DTH reactivity against the second antigen, burro erythrocyte (BRBC), when the conjugate FGG-BRBC is present in the cultures. The requirement for physical linkage between the two antigens has been demonstrated by the observation that DTH to BRBC is induced when the

conjugate FGG-BRBC is present and not when BRBC and FGG are given as uncoupled molecules.

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