

GRAFT-VS.-HOST REACTION IN TISSUE CULTURE*

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Several years ago our laboratory described procedures for eliciting and detecting a graft-vs.-host reaction in tissue cultures (1, 2). In direct analogy to the in vivo system, we demonstrated the enlargement of explanted fragments of neonatal F₁ spleen can be obtained when such fragments are exposed to immunocompetent adult parental lymphoid cells. Such in vitro splenomegaly could be observed in 3–4 days, and was accompanied by many of the histological changes typical of in vivo graft-vs.-host disease (2).

Of all the methods now established as in vitro correlates of cellular immunity, the graft-vs.-host reaction seemed to represent perhaps the most accurate reflection of an in vivo cellular immune reaction, yet its potential utility appeared to us to have been almost overlooked (cf. 3–6). Perhaps in part this has been due to the slightly less conventional methods of tissue culture used, in part to the absence of ready quantitation of the response, and in part because of the developmental orientation of the studies in which this technique has been employed (cf. 7–12). Because of the increasing importance of in vitro methods and because of the specific interest in the graft-vs.-host reaction in transplantation studies, we decided to refine the previous procedures for obtaining an in vitro graft-vs.-host reaction, to establish the quantitative potential of the technique, and to apply the new methodology in studying a number of experimental questions.

Materials and Methods

General Methodology.—The experimental procedure is summarized in Fig. 1. A double culture well system (cf. 7) employing a millipore filter assembly and a plastic organ cultures dish is used. Known numbers of test cells (parental) are placed in one of the wells, while an equivalent number of control cells (F₁) is added to the other one. Neonatal (1–2-day old) F₁ animals serve as a source of spleen tissue explants, two fragments, matched for size, being used for each culture dish; one fragment is placed with the test cells while the other is cultured with control cells. After an appropriate incubation period, tritiated leucine is added to the culture medium and the cultures are further incubated for 12–24 h. The amount of incorporation into each of the fragments is then measured, and the ratio of incorporation of the fragment cultured with test cells to incorporation of the fragment cultured with control

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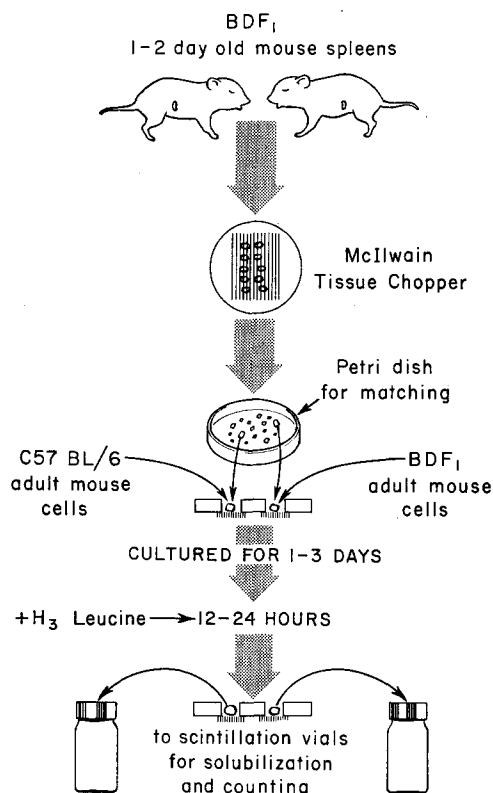


FIG. 1. Schematic diagram of experimental procedure.

cells is determined. In direct analogy to the *in vivo* system, this ratio is defined as the Spleen Index. An index of 1.2 is considered positive.

Cell Suspensions.—C58BL/6 and BDF₁ (C57BL/6 × DBA/2) mice were obtained from our own colony or from ARS Sprague Dawley (Madison). Adult organs were removed, placed in saline solution containing 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.), and prepared as cell suspensions by gentle teasing. Cell counts were obtained by use of a Nuclear Chicago particle size analyzer (Coulter principle) (Nuclear-Chicago Corp., Des Plaines, Ill.) using size discrimination or cell lysis to obtain accurate counts of nucleated cells. Dilutions were then made to permit 5 μ l to contain the appropriate number of cells to be added to each filter well.

Tissue Fragments.—1-2-day old BDF₁ spleens were dissected in saline. 10-20 of these spleens were placed on a chopping plate of a McIlwain tissue chopper and cut into 0.8-mm slices perpendicular to the long axis. Fragments were transferred to saline, then visually matched as pairs for explantation into individual organ culture dishes.

Millipore Filter Assembly.—Sheets of 0.030 inch (ca. 0.8 mm) thick plexiglas (Cadillac Plastics and Chemical Co., Detroit, Mich.) were cut into strips measuring 25 × 6 mm. Two 3-mm holes were drilled into each strip using a drill press and mold to facilitate handling. One side of the strip was marked to facilitate subsequent orientation. A small rectangle of ultra-thin millipore (THWP) filter material was glued to the plexiglas assembly using CD125

(Cadillac) cement. The complete assembly was sterilized in 70% alcohol and rinsed in saline before use.

Tissue Culture.—The culture medium consisted of Eagle's basal medium supplemented with 10% horse serum (Grand Island), 5% chick embryo extract (9-day embryos), gentamycin (50 $\mu\text{g}/\text{ml}$), and mycostatin (50 $\mu\text{g}/\text{ml}$). 1:1 ml of medium was placed into no. 3037 Falcon organ culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.); medium was normally not changed during the experiment. Cultures were incubated in a moist chamber of 95% oxygen and 5% CO_2 at 37°C.

Experimental Procedures.—The filter wells were individually aspirated to remove excess saline after which 5 μl of test (parental) cells were added to one well, while 5 μl of control (F_1) cells were added to the other well. The assembly was then placed in a culture dish containing medium. After all assemblies were filled with cells, spleen fragments were prepared as described above and two fragments were matched for size and placed in the wells. After an appropriate period of incubation (see results) 0.4 μCi of tritiated leucine were added to the medium of each dish and after an additional period of 12–24 h the spleen fragments were removed individually, rinsed in saline, and placed in scintillation vials each containing 0.05 ml of water. 0.5 ml of tissue solubilizer (TS-1, Research Products Int.) was added to each vial; vials were allowed to stand for a minimum of 24 h at room temperature before the addition of 5 ml toluene-PPO-POPOP scintillation fluid for counting. Since spleen fragments were initially matched for size and maintained under identical culture conditions except for the nature of the cells added (i.e., parental vs. F_1) the spleen index could be directly calculated as the ratio of counts of experimental/control for each culture dish.

Chemicals.—Amantadine hydrochloride was obtained as a gift from E. I. Du Pont De Nemours and Co. (Wilmington, Del.), dissolved in standard tissue culture medium, and sterilized by millipore filtration. Mitomycin C (Nutritional Biochemical Corporation, Cleveland, Ohio) was prepared as a stock solution of 0.5 mg/ml. For mitomycin C treatment of cells 1 ml of a cell suspension containing between 5×10^7 and 1×10^8 cells was incubated with 0.05 ml of mitomycin C stock solution for 20 min after which cells were washed three times before explantation. To obtain "cortisone-resistant thymus cells," mice were injected intramuscularly with 2.5 mg hydrocortisone acetate 3 days before utilization.

X irradiation.—Irradiation was performed on a GE Maxitron 300 X-ray machine (General Electric Co., Pleasanton, Calif.), operating at 20 mA, 300 kVp with 0.5-mm Cu and 1.0-mm Al, at a dose rate of approximately 70 R/min, as measured in air by a Victoreen dosimeter (Victoreen Instrument Div., Cleveland, Ohio).

Additional Culture Information.—Several simplifications have been found acceptable although not employed in the course of the present study.

Medium: A mixture of 5% fetal calf serum and 5% horse serum eliminates the need for chick embryo extract.

Gas mixture: Mixtures containing 5% CO_2 and 50% or more oxygen are adequate. In general, smaller fragments require less oxygen.

Assay of radioactivity: Pooling of fragments (experimental vs. control) from several cultures vastly simplifies assay procedure without appreciably affecting statistical significance (cf. Table I). In this case, however, individual culture viability can only be assessed visually before assay. Other labeled amino acids can be substituted for leucine.

Culture dishes and filter assembly: A standard 35-mm culture dish can be used by inserting a glass ring to support the filter mount. A multiple filter assembly containing 3–5 pairs of wells can be constructed for use in such dishes. Care must be taken, however, to maintain fluid level at the correct height to assure culture viability and prevent excessive flattening of the spleen explants. Somewhat thicker (0.040 inch) plexiglass facilitates maintenance of fluid levels in multiple filter assemblies.

RESULTS

Validation of the Methodology.—As a standard effector cell population we employed 10^6 spleen cells, and splenomegaly induced in neonatal explants was assessed by incorporation of tritiated leucine during the 4th day of culture. The detailed results of 16 consecutive experiments involving 148 pairs of explants are shown in Table I. Confidence limits for selected numbers of cultures/experiment have been included in order to provide a frame of reference for the remainder of the experimental results presented in our study. Based on these results, an index of 1.2 or greater was accepted as indicating a positive graft-vs.-host reaction.

Immunocompetence of Lymphoid Cell Populations: Time-Dose Studies.—In order to provide a basis for comparison of various cell populations, the number of cells tested and the time interval during which labeled leucine was available to the cultures were varied. The results of a single experiment with adult spleen cells are shown in Table II. This and other experiments indicate that splenomegaly occurs earlier in cultures containing a higher number of immunocompetent cells and that maximal enlargement of spleen explants fails to occur when the number of immunocompetent cells becomes restricted.

Similar time-dose studies were carried out with thymus cells, with cortisone-resistant thymus cells, with inguinal lymph node cells, with mesenteric lymph node cells, with thoracic duct lymphocytes, and with bone marrow cells. The results of these studies are shown in Tables III–VIII.

Immunosuppression: Effects of Amantadine Hydrochloride, Mitomycin C and X-rays.—Because of the apparent selectivity by which amantadine affects lymphoid cells (13) we decided to examine the effect of this drug on immunocompetence as expressed in the in vitro graft-vs.-host reaction. Concentrations of 100 μg or less did not influence the amount of incorporation of leucine into control cultures and were therefore not considered to have significant toxic effects. The results, shown in Table IX, indicate that 50 $\mu\text{g}/\text{ml}$ is partially inhibitory while concentrations of 75 $\mu\text{g}/\text{ml}$ or higher completely inhibit the graft-vs.-host reaction in vitro. It should be noted, moreover, that since 6×10^4 spleen cells normally cause a measurable response when assayed after 3 days of incubation (cf. Table II) whereas 10^6 cells failed to do so in 75 μg amantadine-containing media, immunosuppression must have been in excess of 95% in these cultures. We did not, however, test other cell concentrations.

Because of the known inhibitory effects of mitomycin C in the mixed lymphocyte reaction (14), we studied the effect of pretreatment of adult spleen cells with mitomycin C on their subsequent ability to induce a graft-vs.-host reaction in vitro. The results, shown in Table X, indicate that mitomycin C is an effective inhibitor of immunocompetent spleen cells as measured in our system.

To examine the degree of radiation sensitivity of immunocompetent spleen cells, adult donor animals were given total body irradiation 24 h before re-

TABLE I
Ability of 10⁶ C57BL/6 Spleen Cells to Induce Splenomegaly in Explanted Neonatal BDF₁ Spleen Fragments as Measured by Incorporation of [³H]leucine on the 4th Day of Culture (16 Consecutive Experiments)

Exp.	No. of cult.	Individual spleen indexes	Mean	SE	SD
1	16	1.25; 1.58; 1.25; 1.71; 1.34; 1.49; 1.40; 1.07; 1.50; 1.30; 1.49; 1.34; 1.42; 1.40; 1.89; 2.35	1.49	.08	.30
2	6	1.40; 1.07; 0.93; 1.37; 1.43; 1.50	1.28	.09	.23
3	8	1.25; 1.48; 1.33; 1.86; 1.52; 1.35; 1.98; 1.63	1.55	.09	.26
4	20	1.21; 1.32; 1.53; 1.75; 1.37; 1.24; 1.81; .92; 1.54; 1.34; .85; 1.25; 1.59; .94; 1.28; 1.07; 1.49; 1.69; 1.30; 1.17	1.33	.06	.27
5	4	1.47; 1.42; 1.64; 0.67*	1.30	.25	.43
6	8	2.05; 1.31; 1.07; 1.48; 1.59; 2.15; 1.64; 1.84	1.64	.13	.36
7	17	1.73; 1.61; 0.99; 1.70; 1.68; 1.52; 0.75; 1.54; 1.67; 1.54; 1.28; 2.28; 1.40; 1.75; 1.74; 1.35; 2.44	1.59	.10	.40
8	10	1.22; 2.02; 1.91; 1.13; 1.82; 1.82; 1.31; 1.78; 1.24; 1.32	1.56	.11	.34
9	8	1.16; 1.51; 0.75; 1.42; 1.53; 1.42; 1.54; 1.12	1.31	.10	.28
10	10	1.60; 1.36; 1.68; 0.99; 1.64; 1.22; 1.41; 1.70; 2.57; 0.97	1.51	.15	.46
11	8	1.47; 1.15; 1.23; 1.02; 1.18; 1.88; 1.48; 1.27	1.34	.10	.27
12	5	1.44; 1.27; 1.39; 1.18; 1.36	1.33	.04	.10
13	8	1.50; 1.18; 1.65; 2.19; 1.35; 1.76; 1.86; 1.61	1.64	.11	.31
14	4	1.85; 2.18; 1.44; 1.24	1.68	.21	.42
15	8	1.53; 0.97; 1.47; 1.27; 0.93; 1.71; 1.57; 1.11	1.32	.10	.29
16	8	1.79; 1.59; 1.78; 1.50; 1.47; 1.18; 1.92; 1.17	1.55	.10	.28
* probably reversed					
All experiments: 148 cultures			1.47	.03	.33
Mean of means			1.46	.04	.14
Confidence limits					
5 cultures					
9 cultures					
16 cultures					
50%			0.10	0.07	0.05
80%			0.19	0.14	0.11
90%			0.24	0.18	0.14
95%			0.29	0.22	0.16

TABLE II
Ability of C57BL/6 Spleen Cells to Induce Splenomegaly in Explanted BDF₁ Neonatal Spleen Fragments

Time of labeling (h after incubation)	Spleen index			
	$1.0 \times 10^{6*}$	$2.5 \times 10^{5*}$	$6.0 \times 10^4*$	$1.5 \times 10^4*$
24-36	1.09	1.04	0.93	0.93
36-48	1.24	1.05	0.96	1.13
48-60	1.45	1.34	0.97	1.04
60-72	1.60†	1.88	1.20	1.07
72-84	1.29	1.36	1.34	1.17

Each index represents six cultures/group. 1.2 is considered positive.

* Number of cells.

† Five cultures.

TABLE III
Ability of C57BL/6 Thymus Cells to Induce Splenomegaly in Explanted BDF₁ Neonatal Spleen Fragments

Time of labeling (h after incubation)	Spleen index		
	$8.0 \times 10^{6*}$	$2.0 \times 10^{6*}$	$5.0 \times 10^5*$
24-36	0.96	1.01	0.95
36-48	1.01	1.00	0.89
48-60	1.17	1.07	1.23‡
60-72	1.34	1.02	1.05
72-84	1.45§	1.33‡	1.00‡
84-96	1.93	1.48	1.08

Each index represents six cultures/group.

* Number of cells.

‡ Five cultures.

§ Four cultures.

TABLE IV
Ability of Thymus Cells from Cortisone-Treated C57BL/6 Mice to Induce Splenomegaly in Explanted BDF₁ Neonatal Spleen Fragments

Time of labeling (h after incubation)	Spleen index		
	$1.0 \times 10^{6*}$	$2.5 \times 10^{5*}$	$6.0 \times 10^4*$
24-36	1.05	1.00	0.97
36-48	1.35	1.08	1.12
48-60	1.44	1.32	1.08
60-72	1.46	1.65	1.00
72-84	1.77	1.58	1.19

Each index represents five cultures/group.

* Number of cells.

TABLE V

Ability of C57BL/6 Inguinal Lymph Node Cells to Induce Splenomegaly in Explanted BDF₁ Neonatal Spleen Fragments

Time of labeling (h after incubation)	Spleen index			
	1.0×10^6 *	2.5×10^5 *	6.0×10^4 *	1.5×10^4 *
12-24	1.14	1.11	1.13	0.93
24-36	1.15	1.14	1.03	1.15
36-48	1.17	1.02	1.09	1.06
48-60	1.21	1.10	1.16	1.10
60-72	1.42	1.23	1.24	1.01
72-84	1.35	1.25	1.18	1.18

Each index represents 10 cultures/group, two experiments.

* Number of cells.

TABLE VI

Ability of C57BL/6 Mesenteric Lymph Node Cells to Induce Splenomegaly in Explanted BDF₁ Neonatal Spleen Fragments

(Time of labeling (h after incubation)	Spleen index			
	1.0×10^6 *	2.5×10^5 *	6.0×10^4 *	1.5×10^4 *
24-36	1.12	1.11	1.06	1.12
36-48	1.04	1.16	1.17	0.99
48-60	1.37	1.43	1.13	0.98
60-72	1.54	1.18	1.21	1.07
72-84	1.17	1.43	1.16	1.23

Each index represents six cultures/group.

* Number of cells.

TABLE VII

Ability of C57BL/6 Thoracic Duct Lymphocytes to Induce Splenomegaly in Explanted BDF₁ Neonatal Spleen Fragments

Time of labeling (h after incubation)	Spleen index		
	1×10^6 *	2.5×10^5 *	6.0×10^4 *
24-36	1.34‡	1.29§	1.27‡
36-48	1.41‡	1.34	1.18
48-60	2.03‡	1.26	1.20
60-72	1.99‡	1.65	1.48
72-84		1.60	1.26

* Number of cells. F₁ control cells used in 60% of cultures only. Presence of control thoracic duct lymphocytes did not influence spleen index.

‡ 5 cultures.

§ 3 cultures.

|| 10 cultures.

TABLE VIII
Ability of C57BL/6 Bone Marrow Cells to Induce Splenomegaly in Explanted BDF₁ Neonatal Spleen Fragments

Time of labeling (h after incubation)	Spleen index		
	8.0×10^6 *	2.0×10^6 *	5.0×10^6 *
36-48	0.95	0.96	1.16
48-60	0.92	1.07	1.13
60-72	1.03	0.97	1.16
72-84	1.15	0.89	1.06
84-96	N.V.‡	0.96	1.15

* Number of cells.

‡ Control cultures show inhibition of incorporation of [³H]leucine.

TABLE IX
Effect of Amantadine Hydrochloride on the Ability of 10^6 C57BL/6 Spleen Cells to Induce Splenomegaly in Explanted BDF₁ Neonatal Spleen Fragments (Results of Three Experiments)

Concentration of amantadine HCl	Number of cultures	Spleen index	± SE
μg			
Control	29	1.56	±0.07
10	15	1.63	±0.11
25	15	1.68	±0.12
50	21	1.21	±0.05
75	15	1.00	±0.04
100	8	0.91	±0.08
150	7	1.05*	±0.05

Cells Labeled from 72 to 96 h.

* Reduced incorporation of [³H]leucine in control cultures indicates general toxicity at this concentration.

TABLE X
Effect of Mitomycin C on the Ability of C57BL/6 Spleen Cells to Induce Splenomegaly in BDF₁ Neonatal Spleen Explants

Number of cells	Spleen index	
	+ Mitomycin C	Control
1.0×10^6	1.08	1.41
5.0×10^5	1.12	1.34
2.5×10^5	1.16	1.31

Each index represents eight cultures/group. Cells labeled from 48 to 72 h.

removal of their spleens. The results are shown in Table XI. Again, referral to Table II provides the necessary information for calculating the effective immunosuppression achieved by various doses of X-irradiation. In addition to the experiments shown in Table XI, we examined the effect of various doses

TABLE XI

Effect of X-Irradiation on the Subsequent Ability of C57BL/6 Spleen Cells to Induce Splenomegaly in Neonatal BDF₁ Spleen Explants

Dose of X-ray	Spleen index		
	1×10^6 *	5×10^5 *	2.5×10^5 *
0 R	1.47	1.45	1.26
250 R	1.31	1.09	1.08
500 R	0.90	1.04	
750 R	1.16		
1,000 R	0.88		

Each index represents 10 cultures/group. Cells labeled from 48 to 72 h.

* Number of cells.

of X-rays on spleen cell suspensions just before their being placed in culture wells; the results were comparable to those achieved with irradiation in vivo.

Effect of Preimmunization on Spleen Effector Cells.—To study the effect of preimmunization in our system, 10^8 adult DBA spleen cells were injected intraperitoneally into adult C57BL/6 mice 5–7 days before spleen cells from such animals were used in the in vitro test system. The results shown in Table XII indicate that while preimmunization results in increased cytotoxicity at high cell densities (10^6 cells/filter well) it does not increase the efficiency of low densities of spleen cells to induce splenomegaly in vitro.

We next examined the effect of preimmunization on radiation sensitivity of the effector cell (7). Preimmunized or control animals were given 500 R 24 h before their spleen cells were used for in vitro assessment of immunocompetence (cf. Table XI). The results are shown in Table XIII, and indicate that preimmunization does have a protective effect on radiation sensitivity of the immunocompetent spleen cell population.

It could be argued, however, that preimmunization enhanced the response to non-*H-2* antigens, and it was this response that was being measured in the experiments involving preimmunization of C57BL/6 animals with DBA cells. We therefore repeated the experimental procedures, employing C57BL/10 animals preimmunized with spleen cells from C57BL/10 D2 mice. The results are shown in Table XIV, and indicate that the protective effect of preimmunization on radiation sensitivity of immunocompetent cells can be seen in a congenic system in which only the major histocompatibility complex is dissimilar.

*Graft-vs.-Host Reaction across Weak Non-*H-2* Barriers.*—To examine the sensitivity of the in vitro graft-vs.-host reaction to detect weak histocompatibility differences the strain combination of BALB/C and DBA/2 was used. For these experiments neonatal spleen explants were matched in groups of three fragments to permit the same control cultures to be used in assessing immunocompetence of cells from both parental sources. At the same time,

TABLE XII

Effect of Preimmunization with DBA Cells on the Ability of C57BL/6 Spleen Cells to Evoke a Graft-vs.-Host Reaction against BDF₁ Neonatal Spleen Explants

Number of cells	Time of labeling	Spleen index	
		Preimmunized	Normal
	<i>h</i>		
1×10^6	24-48	1.03	1.25
	48-72	0.81	1.37
2.5×10^5	24-48	1.05	1.04
	48-72	1.37	1.38
6.0×10^4	24-48	1.18	1.08
	48-72	1.20	1.21
3.0×10^4	48-72	1.05	0.98
	72-96	1.11	1.04
1.5×10^4	24-48	1.05	0.97
	48-72	1.11*	1.08*
	72-96	1.15	1.07
7.5×10^3	48-72	0.93	1.09
	72-96	0.84	1.06

Each index represents six cultures/group.

* 12 cultures.

TABLE XIII

Effect of Preimmunization against DBA on the Ability of Irradiated C57BL/6 Spleen Cells to Induce Splenomegaly in Explanted Neonatal BDF₁ Spleen Fragments

	Spleen index		
	1×10^6 *	5×10^5 *	2.5×10^5 *
Preimmunized + 500 R X-ray	1.48	1.34	1.32
500 R X-ray only	1.03	0.90	

Each index represents 10 cultures/group. Cells labeled from 48 to 72 h.

* Number of cells.

TABLE XIV

Effect of Preimmunization against B10·D2 on the Ability of Irradiated C57B/10 Spleen Cells to Induce Splenomegaly in Explanted Neonatal BDF₁ Spleen Fragments

Treatment	Spleen index		
	1×10^6 *	5×10^5 *	2.5×10^5 *
Preimmunized + 500 R X-ray	1.35	1.29	1.23
500 R X-ray only	1.12	1.12	
Untreated	1.30‡	1.49‡	

Each index represents 18 cultures/group. Cells labeled from 48 to 72 h.

* Number of cells.

‡ 8 cultures.

the cell suspensions were tested for their ability to induce a graft-vs.-host reaction in vivo. The results are shown in Table XV, and indicate that a weak but clearly detectable response could be obtained in vitro.

TABLE XV
Ability of Spleen Cells from DBA/2 or BALB/C Mice to Induce a Graft-vs.-Host Reaction against F₁ (BALB/C × DBA) In Vivo or In Vitro

	Cells tested	No. of animals	Mean spleen index
In vivo*	BALB/C × DBA/2	9	(1.00)
	BALB/C	11	0.87
	DBA/2	11	0.96
In vitro‡		<u>No. of cultures</u>	
	BALB/C	17	1.21
	DBA/2	17	1.20

* 5×10^6 cells injected into 2-3-day old mice, assayed 9 or 11 days later (two experiments).

‡ 10^6 cells/culture, three-way match, each set including one control culture of BALB/C × DBA cells; labeled from 48 to 72 or 72 to 96 h (two experiments).

DISCUSSION

A primary purpose of our report has been to validate the in vitro graft-vs.-host reaction as an experimental system. We feel that this has been accomplished. We have demonstrated that a graft-vs.-host reaction can be induced by appropriate numbers of spleen, lymph node, thoracic duct, or thymus cells, that the radiation sensitivity of immunocompetent cells measured in vitro is similar to that seen in vivo, that cortisone treatment leaves a highly competent thymus cell population, and that preimmunization does not appear to change the number of effector cells active across strong *H-2* barriers.

A number of findings of our study, however, require further discussion. Our observation that bone marrow cells do not cause a graft-vs.-host reaction in vitro may have several possible explanations. Of these the simplest one is to ascribe their failure to technical reasons. If the number of immunocompetent cells in the bone marrow is lower than it is in the untreated thymus (cf. 15) then the concentration of bone marrow cells required to induce a graft-vs.-host reaction in vitro is too great to permit long-term viability of the cultures. Indeed we found that at a concentration of 8×10^6 cells/culture viability was only marginal by the end of the 3rd day of culture. The use of cell separation techniques to obtain a higher yield of presumed immunocompetent bone marrow cells should permit test of this explanation. On the other hand, it may well be that bone marrow cells must undergo further differentiation which

could take place in vivo, but may simply not occur in vitro or be achieved too slowly to be recognized during the 3–4-day culture period. In fact, embryonic liver cells appear to follow this pattern (8, 9).

Our observation that amantadine inhibits the graft-vs.-host reaction in vitro becomes particularly interesting in the light of the observation by Mardiney and Bredt (13) that amantadine interferes with immunological recognition but not with the response of lymphocytes to phytohemagglutinin. Taken together with the observation that mitomycin C inhibits the graft-vs.-host reaction it suggests to us that our in vitro system may provide a most sensitive tool for distinguishing between general and more specific immunosuppressive effects.

To us there has long been some ambiguity concerning the effect of preimmunization on effector cells in the graft-vs.-host reaction. For example, while Simonsen's "factor of immunization" concept has stressed that preimmunization fails to increase significantly the number of cells responsive across strong *H-2* barriers (16, cf. also 15), our own earlier in vitro studies had indicated that preimmunization increased both the ability of cells to induce splenomegaly across an intervening millipore filter (2) and that it reduced the radiation sensitivity of cells competent to cause a graft-vs.-host reaction (7). The experiments we now report indicate that in direct confirmation of in vivo studies we cannot detect an effect of preimmunization under normal conditions, yet after irradiation the effect of that preimmunization is readily apparent.

We had considered the possibility that preimmunization increased the radioresistance and immunocompetence of cells responding to non-*H-2* antigens, since in that case one would expect a factor of immunization considerably greater than 1. However, since we obtained essentially identical results in a congenic system differing only at the *H-2* complex this explanation is inadequate. It will be interesting to determine whether preimmunization changes the effector cell population in a qualitative way or whether, perhaps, preimmunization leads to the production of a residual memory cell population that can lead to restitution following irradiation.

To assess the ability of the in vitro system to detect differences not involving the *H-2* locus we selected a strain combination (BALB/C and DBA/2) in which a discordance between in vitro and in vivo assays had already been described (18). While this particular strain combination was known not to cause splenomegaly in vivo, Festenstein had observed that stimulation in mixed leukocyte culture was as strong between BALB/C and DBA as it was between BALB/C and the *H-2* incompatible strain CBA. When we assayed DBA and BALB/C cells using the technique (14) of one-way mixed leukocyte culture, we found that BALB/C cells were stimulated as well by DBA/2 as by CBA cells, and even DBA/2 cells responded almost as strongly (80%) to BALB/C as to CBA cells. At the same time we too were unable to show a graft-vs.-host reaction in vivo using the Simonsen assay. In our in vitro system,

on the other hand, while both DBA and BALB/C cells were able to induce splenomegaly, the response was consistently weak. Since most standard tests of transplantation would have predicted such a weak reaction, and since recently it has been shown that this strain combination causes a weak graft-vs.-host reaction as measured by local lymph node enlargement (19), it would appear that our in vitro assay of the graft-vs.-host reaction may be more representative of the strength of immunological incompatibility than either the in vivo splenomegaly assay or the in vitro mixed leukocyte system.

As part of the present study we initiated an extensive series of experiments (436 cultures) in which we attempted to study synergistic interactions between inguinal lymph node cells and normal thymus, regenerating thymus, or thymus-derived spleen cells. By use of a variety of cell concentrations and labeling times we felt that the in vitro system would provide an ideal opportunity for characterizing the nature of synergism in the graft-vs.-host reaction. Nevertheless, we were unsuccessful in demonstrating any significant degree of cell interactions in our experiments. However, our test system utilized C57BL/6 effector cells tested against BDF₁ neonatal spleen explants, and when we attempted to show synergism in vivo we were unable to do so in this strain combination. Since the studies after which we modelled our experiments were carried out with BALB/C effector cells reacting against BALB/C × C57BL/6 hybrids, it now becomes of paramount importance to repeat our experiments using the strain combinations for which synergism had been previously reported (17).

The described in vitro assay of the graft-vs.-host reaction certainly requires more effort and care than is needed for the in vivo assessment; for this reason some of the advantages of the in vitro system should be emphasized. The system is highly reproducible, internally controlled, and readily expressed in quantitative terms. Attrition due to maternal neglect or host variability is eliminated. A relatively short period of time (2-3 days) is required before a positive reaction can be observed. There is a high degree of sensitivity both as seen in combinations not involving the *H-2* locus and as evidenced by the detection of responses given by low numbers of cells. Multiple cultures can be set up; there are inherent controls for viability; cells can be added or removed at will; and the system lends itself to the use of immunosuppressants, antisera, and the use of sequential labeling procedures. Taken together these features would seem to suggest that the in vitro graft-vs.-host reaction may be particularly useful for studies of differentiation of immunocompetent cells, for analysis of specific cellular subpopulations, and for a critical attack on the questions of cell interactions in cellular immunity.

SUMMARY

The primary purpose of this study has been to validate the in vitro graft-vs.-host reaction as an experimental system. Time-dose studies have been presented for cells obtained from spleen, thymus, cortisone-treated thymus,

inguinal lymph node, mesenteric lymph node, thoracic duct, and bone marrow cells. Both the degree of splenomegaly and the onset of spleen enlargement were found to be dependent on the number and source of cells tested.

The effect of several immunosuppressive agents was examined. Amantadine was found to suppress completely the graft-vs.-host reaction in vitro when present at a concentration of 75 $\mu\text{g}/\text{ml}$. Pretreatment of effector cells with mitomycin C prevented their subsequent ability to cause a graft-vs.-host reaction. The effect of X irradiation on immunocompetence of spleen cells in vitro paralleled the known effect of irradiation on in vivo immunocompetence.

Preimmunization did not increase the number or effectiveness of immunocompetent cells when measured under standard in vitro conditions. Preimmunization did, however, permit persistence of immunocompetence after immunosuppressive doses of X irradiation. Studies using congenic lines, moreover, indicated that the preimmunization effect could be demonstrated in strain combinations differing only in factors determined by the *H-2* complex of genes.

A weak graft-vs.-host reaction could be detected in strain combinations not involving differences at the *H-2* locus. The potential of the in vitro graft-vs.-host reaction as a highly reproducible, quantifiable, internally controlled, and experimentally accessible system for study of such critical problems as cell differentiation and cell interactions is discussed.

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