IMMUNOLOGICAL PROPERTIES OF SUBCELLULAR RAT LYMPHOCYTE PREPARATIONS

Primary Allogeneic Stimulation In Vitro by Fractions Containing Ia (RT1-B), but Not RT1-A Antigens*

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The proliferative response of lymphocytes during allogeneic mixed-lymphocyte cultures is stimulated principally by antigens of a distinct region of the major histocompatibility complex (MHC)[1] (1–4). Reexposure of primed alloimmune lymphocytes to subcellular preparations or liposome-incorporated antigens obtained from the immunizing strain, but not MHC-disparate strains, stimulates an accelerated generation of cytotoxic T lymphocytes (CTL) (5–10). However, primary mixed-lymphocyte reponses (MLR) and CTL generation have been reported to occur only when stimulated by intact metabolically active allogeneic lymphocytes (11, 12). Although inhibition of DNA-synthetic mechanisms by x-irradiation or mitomycin C does not impair the ability of intact cells to stimulate, exposure to thermal or ultraviolet energy will destroy stimulating ability (13). Hence, the requirements for MLR stimulation have appeared to require a metabolic or cell-surface configurational state not available when membrane fragments or isolated Ia molecules are employed. This circumstance has limited studies on the relationship of MLR-stimulating and serologically defined Ia specificities. MLR stimulation and expression of Ia-like antigens are encoded by the RT1-B region of the rat MHC (14–16). Rat Ia antigens are structurally similar to those of the mouse (17, 18), principally expressed on B lymphocytes and macrophages. We have reported that 5–6% of rat thymocytes are Ia positive and stimulate in MLR (19). We now present evidence that membrane preparations from thymic lymphocytes induce primary MLR-like stimulation when cultured with allogeneic rat T cells. These preparations exhibit Ia antigenic properties but lack detectable RT1-A (Ag-B) alloantigens.

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[1] *Abbreviations used in this paper:* Ad, nylon-wool adherent; CDC, complement-dependent cytotoxicity; CTL, cytotoxic T lymphocytes; EAI, EA-rosette inhibition; FCS, fetal calf serum; Fm, membrane fragment; Ir, immune response; MHC, major histocompatibility complex; MLR, mixed-lymphocyte response; NAd, nylon-wool nonadherent; RBC, erythrocytes; SE, supernatant extract; TdR, thymidine.

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Materials and Methods

Animals. Inbred male rats, weighing ~200 g; WF(Ag-B^2, $RT1^u$), BN(Ag-B^3, $RT1^n$), LEW(Ag-B^1, $RT1^l$), BUF(Ag-B^6, $RT1^b$), and F344(Ag-B^1, $RT1^{lv1}$) were purchased from Microbiological Associates, Walkersville, Md.; as were the hybrids (WF × BN)F_1 and (LEW × BN)F_2.

Materials. RPMI-1640 cell culture medium and heat-inactivated fetal calf serum (FCS) were purchased from Associated Biomedic Systems, Inc., Buffalo, N. Y. Tritiated thymidine ([^{3}H]TdR) (sp act: 6.7 Ci/mM or 20.0 Ci/mM) and [^{14}C]thymidine ([^{14}C]TdR [sp act: 40.8 mCi/mM]) were purchased from New England Nuclear, Boston, Mass. Ox erythrocytes were purchased from Baltimore Biological Laboratory, Cockeysville, Md. All cell culture experiments were performed in flat-bottomed microtiter plates purchased from Cooke Engineering Co., Alexandria, Va. A MashII cell harvesting apparatus, obtained from Microbiological Associates, was used to harvest cell cultures. Nonradioactive thymidine (lot 010156) was obtained from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.

Preparation of Cellular Fragments (Fm) and Supernatant Extract (SE). Fm were prepared by a slight modification of Davies's technique (20). Lymphocytes obtained from rat spleen or thymus were suspended in hypotonic saline (0.8%), submitted to freeze-thaw lysis, and centrifuged at 600 *g* for 30 min. The supernate was retrieved. The pellet was resuspended in 0.7% NaCl solution, resubmitted to freeze-thaw lysis, and centrifuged again at 600 *g* for 15 min. Both supernates were then pooled and centrifuged at 60,000 *g* for 90 min. The resultant pellet, containing Fm, was resuspended in RPMI-1640 medium. The hypotonic supernate, or SE was dialyzed overnight at 4°C against RPMI-1640. Fm and SE were both stored at −70°C.

MLR. Rat spleen cells were divided into nylon-wool adherent (Ad) and nonadherent (NAd) populations as described by Handwerger and Schwartz (21). 1 million LEW responder NAd cells in 0.1 ml were mixed with an equal concentration of mitomycin C-treated BN or (LEW × BN)F_1-stimulating cells in flat-bottomed microtiter plates utilizing four to eight replicates. The plates were cultured in a CO_2 incubator and pulsed with 1 μCi/well of [^{3}H]TdR 4 h before harvest. The cells were harvested onto fiberglass filters with a Mash II apparatus. Dried fiberglass filters were counted in scintillation fluid with a Beckman beta-counter LS-330 (Beckman Instruments, Inc., Fullerton, Calif.).

Fm MLR- and SE MLR-like Reactions. Fm and SE were quantitated in cell-equivalents, i.e., the amount of material added to a culture was expressed in terms of the number of lymphocytes utilized to prepare the Fm or SE. Responder rat lymphocytes were incubated with serial dilutions of Fm and SE preparations ranging from 100:1 to 1:1 ratios of cell equivalents to responder cells in 0.2 ml total vol.

Alloimmune Sera. Heat-inactivated (56°C for 30 min) LEW anti-BN hyperimmune neat and platelet-absorbed sera were prepared as previously described (14): after rejection of BN skin grafts, LEW rats received serial injections of pooled BN lymphocytes at 2-wk intervals. Platelet-absorbed sera were prepared by absorption of LEW anti-BN serum with BN platelets. (WF × BN)F_1 anti-LEW hyperimmune serum was prepared as above by immunization of (WF × BN)F_1 rats with a LEW skin graft followed by biweekly intraperitoneal injections of pooled LEW lymphocytes at 2-wk intervals. These antisera immunoprecipitate typical Ia-like molecules upon polyacrylamide gel electrophoresis analysis (18).

EA-Rosette Inhibition (EAI) Assay. Fc receptor-bearing rat spleen cells form EA rosettes with ox erythrocytes (RBC) sensitized with the IgG fraction of rabbit anti-ox RBC antiserum (22). EA-rosette-forming cells were enumerated by incubating the spleen cells (5 μl of BN or LEW cells at 10^7/ml in buffered RPMI-1640), with 5 μl of 2% ox EA and 3 μl of ox-RBC-absorbed FCS in duplicate wells of round-bottomed microtiter plates after centrifugation at 500 *g* for 10 min at 4°C. A rosette was defined as three or more RBC bound to a mononuclear cell. These rosettes were gently resuspended with 10 ml of buffered RPMI-1640. The percentage of rosette-forming cells was determined after a minimum of 200 cells were counted in a Newbauer counting chamber (Neubauer Mfg., Minneapolis, Minn.) using acridine orange- (23) coated cover slips with simultaneous ultraviolet light and phase-contrast illumination. In rosette-inhibition experiments, LEW or BN cells were preincubated for 45 min at 4°C with test alloantiserum before assessment of rosette formation. The percentage of EAI was calculated using the following formula:

$$\text{Percent EAI} = 100 - \left(\frac{\text{percent EA rosettes in test serum}}{\text{percent EA rosettes in RPMI-1640}}\right) 100.$$

Complement-dependent Cytotoxicity Assay. The complement-dependent cytotoxicity (CDC) of absorbed (WF × BN)F_1 anti-LEW serum was tested on unseparated LEW lymph node cells, Ad, and NAd populations as described previously (14, 24, 25). Results are expressed as counts per minute ± 1 SD of ^{51}Cr release.

TdR Suicide Protocol. In a modified version of the technique described by Moorhead (26), WF lymph node cells (25×10^6) were incubated in a modified Marbrook culture chamber with either 25×10^6 mitomycin-treated LEW thymocytes or 2×10^8 cell equivalents of LEW SE or Fm in culture medium containing RPMI-1640, 10% heat-inactivated FCS, 50 μM 2-mercaptoethanol, and 0.005 M Hepes buffer at 37°C in 10% CO_2. Those cultures were harvested after 72 or 96 h of incubation. The cells were washed twice and adjusted to 5×10^6/ml in RPMI-1640 with 10% FCS. A 1.0-ml aliquot was incubated with 10 μCi of [^{3}H]TdR for 60 min at 37°C in 10% CO_2, while another aliquot (untreated cells) was incubated without [^{3}H]TdR. Subsequently both aliquots were resuspended in 10 ml of RPMI-1640 that contained 100 μl/ml of unlabeled TdR and incubated for an additional 30 min. Each aliquot was washed twice, the cells counted and a series of secondary cultures were prepared in flat-bottomed microtiter trays with mitomycin-treated LEW thymocytes, mitomycin-treated BN thymocytes, LEW Fm, or LEW SE at concentrations previously shown to give an optimal proliferative response in MLR. Secondary cultures were harvested after 72 h. The untreated cell cultures were pulsed 4 h before harvesting with 1.0 μCi [^{3}H]TdR/well, and the hot pulse cultures were harvested following a 4–10 h pulse with 0.2 μCi/well of [^{14}C]TdR.

Genetic Segregation of Fm/SE Proliferative Reponse. MHC antigens of 19 F_2 progeny of LEW × BN matings (LEW × BN)F_2 were determined by RBC hemagglutination and the capacity to stimulate LEW or BN cells in MLR. (*a*) RBC hemagglutination: heparinized blood was obtained by tail vein puncture and was used with BN anti-LEW and LEW anti-BN hyperimmune sera to type each animal for the RT1-A locus (14, 15, 27). (*b*) MLR and Fm/SE MLR-like reactions: cervical lymph nodes were sterilely removed from each animal. These lymph node cells were cultured in flat-bottomed microtiter trays as described above, using LEW lymph node cells (mitomycin treated), LEW Fm, LEW SE, BN lymph node cells (mitomycin treated), BN Fm, or BN SE. A positive MLR response was defined as a proliferative response (counts per minute of TdR uptake − machine background) significantly greater than ($P < 0.05$) the proliferative response of unstimulated cells, and was always severalfold greater.

Absorption of Alloantisera with Fm and SE. Aliquots of test sera were incubated with Fm or SE at 37°C for 45 min and then overnight at 4°C. The mixtures were then sedimented at 100,000 *g* for 1 h, and the supernate harvested for further use.

Rat Immunization Protocol. LEW rats were immunized with BN Fm or SE, using 10^8 cell equivalents per rat each time. Day 1: footpad injection of BN Fm or SE mixed with equal volume of complete Freund's adjuvant. Day 20: booster with intraperitoneal injection of Fm or SE. Day 35: second intraperitoneal booster with Fm or SE. Day 40: testing for anti-BN alloantibody in sera of immunized animals.

Removal of Phagocytic Cells. NAd spleen cells were further depleted of phagocytic cells in some experiments by incubation with carbonyl iron, and removing iron phagocytic cells by running the cell suspension through a magnetic field (28).

Results

MLR-like Phenomenon. The interaction of allogeneic but not syngeneic thymus Fm or SE with unprimed lymphocytes results in enhanced [^{3}H]TdR incorporation. This stimulation is seen when NAd LEW spleen cells are cultured with BN thymus-derived Fm or SE. Table I shows representative data from a series of 10 experiments. The response is immunologically specific, because BN fragments do not stimulate BN or (LEW × BN)F_1 cells. Fm and SE from other strains stimulate RT1 disparate strains appropriately (Table II). F344 ($RT1^{lv1}$) and LEW ($RT1^l$) share A region antigens

TABLE I
Fm MLR and SE MLR Reactions

Responder cell*	Stimulator‡	[^{3}H]TdR§	Stimulation index‖
LEW spleen cells	LEW spleen cells*	731 ± 80	—
LEW spleen cells	LEW thymus Fm	698 ± 122	—
LEW spleen cells	BN thymus cells	10,987 ± 826	15.03
LEW spleen cells	BN thymus Fm	4,750 ± 642	6.49
BN spleen cells	BN thymus Fm	1,906 ± 461	—
BN spleen cells	BN thymus cells	2,006 ± 302	—
LEW spleen cells	LEW spleen cells	1,895 ± 194	—
LEW spleen cells	BN thymus SE	12,744 ± 459	6.73
LEW spleen cells	BN spleen SE	1,627 ± 352	—
LEW spleen cells	LEW thymus SE	1,761 ± 114	—

* All spleen cells are NAd lymphocytes.
‡ Whole cells used as stimulators are treated with mitomycin C.
§ Thymidine incorporation (cpm) at 72 h ± SD for Fm MLR and at 48 h for SE MLR.
‖ Stimulation index, stimulated counts per minute/base-line counts per minute.

TABLE II
Strain Specificity of Fm- and SE-induced Blastogenesis in Primary MLR-like Reaction

Stimulus	Responder cells	
	LEW ($RT1^l$)	F344 ($RT1^{lv1}$)
BUF ($RT1^b$) SE	4.2 ± 1.6*	3.1 ± 0.7
BUF ($RT1^b$) Fm	6.0 ± 2.8‡	5.7 ± 1.4
BN ($RT1^n$) SE	6.5 ± 2.2	5.8 ± 2.1
BN ($RT1^n$) Fm	5.4 ± 1.8	7.5 ± 1.2
F344 ($RT1^{lv1}$) SE	1.5 ± 0.8	0.9 ± 0.3
F344 ($RT1^{lv1}$) Fm	ND	ND
LEW ($RT1^l$) SE	1.7 ± 0.6	1.7 ± 0.4
LEW ($RT1^l$) Fm	0.9 ± 0.4	1.2 ± 0.6

* Stimulation index obtained with SE at 48 h: mean ± SD of three different experiments using three different batches of SE, done in four to six replicates.
‡ Stimulation index obtained with Fm at 72 h: mean ± SD of three different experiments using three different batches of Fm, done in four to six replicates.

and the MLR and MLR-related Ia antigens (Ia-1) of the B region, but differ by a non-MLR-related Ia antigen (Ia-2) of the MHC (15). This antigenic difference does not produce significant MLR stimulation with intact cells or fragments.

The MLR-like stimulation is elicited within an optimal ratio of responder cells to allogeneic subcellular fragments. Optimal responder cell to Fm or SE ratios are obtained at Fm dilutions ranging from 1/10 to 1/20 and SE dilutions 1/8 to 1/32 (of 50×10^6 cell equivalent/milliliter stock of Fm or SE) (Fig. 1). The response is seen when each responder cell is cultured with Fm or SE derived from 6–12 thymic lymphocytes (i.e., 6–12 cell equivalents). High doses of Fm were more suppressive of the MLR-like phenomenon than high doses of SE. Although lymph node cell membrane fragments have been reported to be cytotoxic in vitro (29), thymus Fm or SE preparations, even at high concentrations where stimulation no longer occurred, did not induce cytolysis in cocultured cells as assessed by eosin dye exclusion studies.

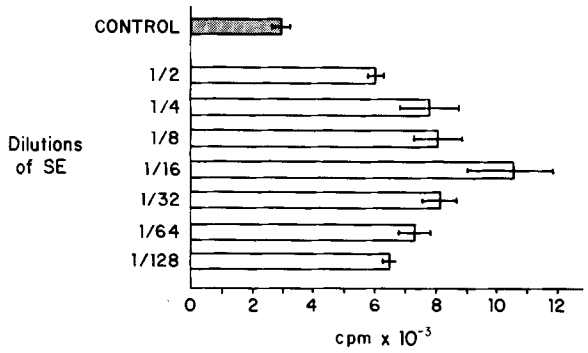

FIG. 1. Dose-response effects of SE MLR-like reaction. The proliferative response of 10^6 NAd spleen cells occurs within an optimal dose range. In this representative experiment peak response is seen with 1/16 dilution of SE at 48 h; other preparations were optimal at 1/8–1/32. (mean ± SD of four to six replicates.)

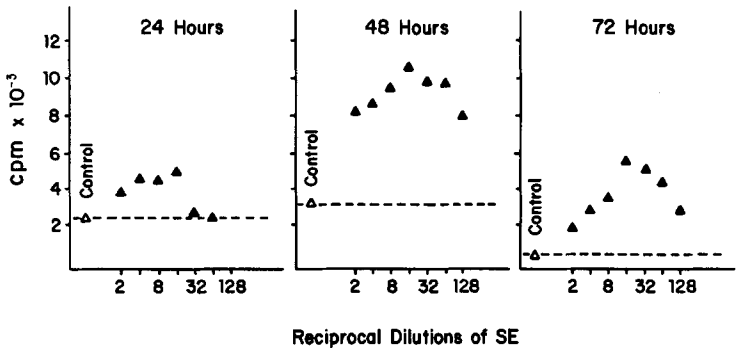

FIG. 2. Kinetics of SE MLR. A positive SE response is seen at 24 h, peaks at 48 h, and is still detectable at 72 h. By 96 h, TdR incorporation returns to base line.

Fm and SE prepared from whole spleen were not stimulatory, and they depressed [^{3}H]TdR incorporation in both syngeneic and allogeneic lymphocytes in a noncytotoxic fashion (data not shown).

The MLR-like response to thymus Fm is first detectable after 48 h of culture and persists through 72 and 96 h of incubation. This kinetic response is identical to one-way cell-cell MLR. In contrast, SE stimulation is evident by 24 h of culture, peaks at 48 h, and is still detectable after 72 but not 96 h (Fig. 2).

The Fm response and the classical one-way MLR require the presence of phagocytic cells. In contrast, the SE response is unaffected by removal of carbonyl iron phagocytic cells. Both cell-cell MLR and Fm response were suppressed by the removal of the phagocytic cells, whereas the SE response was virtually unchanged. (Fig. 3) Consequently, SE promotes early MLR-like effects and bypasses the need for phagocytic cell macrophage participation.

Specificity of Proliferative Response. To examine the relationship of cells responding to allogeneic Fm and SE, the thymidine suicide technique was adopted. This procedure involves the exposure of an antigen stimulated population of cells to [^{3}H]-TdR of very high specific activity. The cells that undergo *de novo* DNA synthesis will

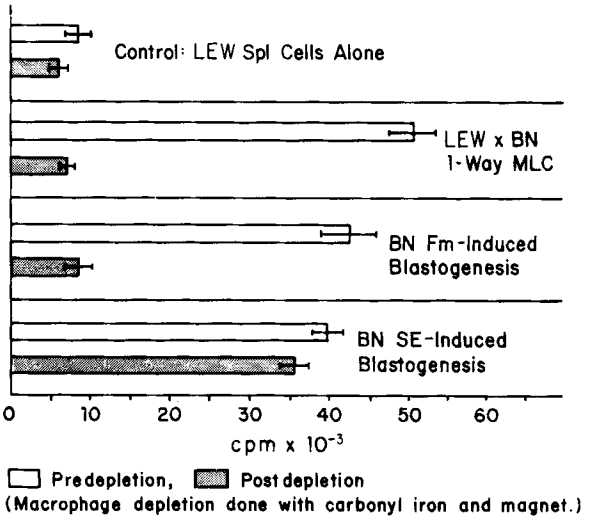

FIG. 3. Effect of macrophage depletion on blastogenesis. TdR incorporation is measured before and after removal of phagocytes. Both cell-cell MLR and the Fm response are suppressed by the macrophage depletion; whereas the SE response is virtually unchanged. (Representative of five experiments, mean ± SD of four to six replicates.)

accrue a lethal intranuclear concentration of radioactivity. Nonproliferating cells are subsequently rescued by addition of unlabeled thymidine. Re-presentation of the initial stimulating antigen to a population that has suffered radiation-induced damage to the responding clones will result in no secondary response. Presenting these cells with different alloantigens, however, should result in a normal proliferative response. The results in Table III show that WF lymph node cells, which are initially stimulated with either LEW thymocytes, LEW SE, or LEW Fm, and then treated with high-dose [^{3}H]TdR, are unable to respond to LEW but are still capable of proliferating in response to BN thymocytes. In contrast, WF lymph node cells that are primed in the absence of lethal doses of [^{3}H]TdR to LEW thymocytes, LEW SE, or LEW Fm and then recultured, are capable of responding to LEW thymocytes, LEW SE, and LEW Fm, as well as the third-party BN cells. This method for selective destruction of alloreactive clones indicates antigenic identity of LEW Fm, LEW SE, and the cell-surface LEW lymphocyte antigens responsible for the classical MLR response.

Linkage of MLR-like Response to RT1 MHC. Additional evidence that the antigenic stimulus for the proliferative response induced by SE and Fm is identical to the MLR phenomenon by intact cells was obtained in genetic segregation studies, testing the response of lymph node cells obtained from (LEW × BN)F_2 animals. Animals were typed by hemagglutination for RT1-A and with Ia-specific antisera upon B cells for RT1-B antigens. MLR typing was performed with homozygous mitomycin-treated BN or LEW lymph node cells. No recombinants between RT1-A and RT1-B were found in the animals studied. When compared to MLR typing with cells, the BN Fm and SE produced virtually identical results (Table IV). The single discrepancy of an *l/l* animal failing to respond to BN Fm is most likely a technical error. Thus, the proliferative phenomenon in the presence of allogeneic fragments has the same genetic requirements for the RT1 linkage as the cell-cell MLR.

TABLE III

*Thymidine Suicide Experiments Showing Identity of MLR-stimulating Antigens on Intact Cells and Membrane Fragments**

Primary culture	Secondary stimulating antigen	Experiment 1		Experiment 2	
		Hot pulse 72 h	Untreated 72 h	Hot pulse 96 h	Untreated 96 h
		cpm $^{14}C \pm 1\ SD$	*cpm* $^{3}H \pm 1\ SD$	*cpm* $^{14}C \pm 1\ SD$	*cpm* $^{3}H \pm 1\ SD$
WF lymph node + LEW thymus (m)	None	73 + 29‡	204 ± 40	40 ± 3	2,901 ± 627
	LEW thymocytes (m)	67 ± 13 (0.9)§	8,912 ± 3,033 (43.7)	72 ± 17 (1.8)	61,126 ± 20,809 (21)
	BN thymocytes (m)	2,338 ± 555 (32.0)	1,319 ± 226 (6.5)	2,008 ± 597 (50.0)	58,883 ± 14,843 (20)
	LEW Fm	34 ± 5 (0.5)	4,021 ± 685 (19.7)	54 ± 17 (1.3)	5,985 ± 640 (2.1)
	LEW SE	36 ± 7 (0.5)	1,938 ± 284 (9.5)	63 ± 19 (1.6)	6,501 ± 798 (2.2)
WF lymph node + LEW Fm	None	218 ± 40	4,097 ± 1434	286 ± 110	58,089 ± 1,873
	LEW thymocytes (m)	243 ± 31 (1.1)	158,902 ± 21,101 (38.8)	181 ± 48 (0.6)	124,248 ± 13,767 (2.1)
	BN thymocytes (m)	1,562 ± 630 (7.2)	73,730 ± 35,756 (18.0)	754 ± 262 (2.6)	96,885 ± 7,778 (1.7)
	LEW Fm	136 ± 29 (0.6)	15,083 ± 2,050 (3.7)	48 ± 3 (0.2)	100,714 ± 3,845 (1.7)
	LEW SE	23 ± 14 (0.1)	30,701 ± 46,779 (7.5)	38 ± 8 (0.1)	116,269 ± 18,571 (2.0)
WF lymph node + LEW SE	None	39 ± 6	2,861 ± 1,405	108 ± 8	25,634 ± 5723
	LEW thymocytes (m)	50 ± 12 (1.3)	51,266 ± 5,362 (17.9)	144 ± 69 (1.3)	89,430 ± 7,295 (3.5)
	BN thymocytes (m)	353 ± 112 (9.0)	87,365 ± 13,226 (30.5)	1,012 ± 345 (9.4)	64,707 ± 4,441 (2.5)
	LEW Fm	9 ± 1 (0.2)	5,415 ± 465 (1.9)	87 ± 39 (0.8)	33,107 ± 1,075 (1.3)
	LEW SE	10 ± 1 (0.3)	6,962 ± 574 (2.4)	73 ± 25 (0.7)	15,620 ± 4,475 (0.6)

* Responding WF lymph node cells after exposure to mitomycin-treated (m) LEW thymocytes, LEW Fm, or SE for 72 h (Exp. 1) or 96 h (Exp. 2) were hot pulsed with 10 μCi. [^{3}H]TdR, washed, and reexposed to the same stimuli and BN thymocytes for an additional 72 h. The secondary cultures were trace pulsed with [^{14}C]TdR or [^{3}H]TdR.

‡ Counts per minute after background subtraction.

§ Stimulation index (experimental counts per minute/counts per minute for none).

TABLE IV

Segregation of MLR-like Response to Membrane Fragments

Stimulator fragments	RT1 genotypes* of responder cells from F_2 progeny			
	n/n	*l/n*	*l/l*	
BN SE	0/4‡	0/9	6/6	$\chi^2 = 38.3$, $P < 0.0005$
BN Fm	0/4	0/9	5/6	$\chi^2 = 31.5$, $P < 0.0005$

* Defined by serologic and MLR typing.

‡ No. positive/No. tested.

Fm and SE Exhibit Ia Antigenicity. Serology: the presence of anti-Ia antibodies in rat alloimmune sera can be demonstrated by specific EAI by target Fc receptor-bearing lymphocytes or by cytotoxicity upon purified B lymphocytes (14, 16, 30). The EAI technique offers the advantage of not being affected by the presence of anti-RT1-A or anti-T cell antibodies, such as anti-AgF (31). Prior absorption of immune sera with Fm or SE obtained from the immunizing strain specifically removes the EA-inhibiting properties of alloimmune sera (anti-RT1-B), whereas hemagglutinating (anti-RT1-A) activity is maintained. Fig. 4 compares both EA rosette-inhibiting properties and hemamagglutinating activity of serial dilutions of a potent LEW anti-BN hyperimmune serum. When absorbed with BN SE, the EA-inhibiting property is virtually

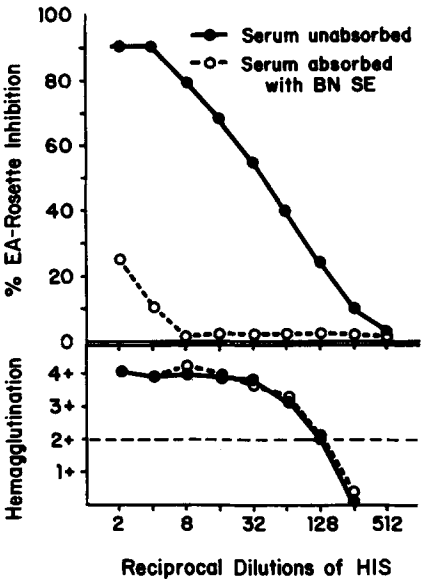

FIG. 4. Effect of SE on hyperimmune alloantisera. Serial dilutions of LEW anti-BN hyperimmune serum (HIS) are each absorbed with a given dose of BN SE (3×10^7 cell equivalents). Although the anti-AgB titer (hemagglutination assay) remains unaltered (lower panel), the anti-Ia titer (EA-rosette inhibition assay) is virtually abolished.

abolished. In contrast, the same absorbed antiserum showed the same hemagglutination titer. Identical results were obtained with Fm. Thus, Fm and SE carry quantities of strain-specific Ia (RT1-B) antigens, although they lack appreciable amounts of RT1-A (Ag-B) specificities.

The CDC technique on purified T and B cells was also employed to assess the effects of antisera absorptions, because Ia antigens are not appreciably expressed on resting T cells. Care must be taken in interpreting results of cytotoxicity data because class I β_2-microglobulin-containing antigens on both T and B cells, and additional differentiation antigens are present on T cells. To demonstrate the presence of Ia antigens and the absence of class I RT1-A antigens in the SE preparation, absorption studies using SE and (WF × BN)F_1 anti-LEW hyperimmune serum were performed. Absorption with either LEW SE or BN SE produced no change in the CDC titer on unseparated or nonadherent-LEW lymph node cells. However, when tested on adherent LEW lymph node cells (Fig. 5) there was an eightfold fall in the CDC titer. Proof that the remaining cytotoxicity in this absorbed serum was attributable to anti-RT1-A activity was obtained by a further absorption with LEW RBC. (Fig. 6) Rat RBC express RT1-A, but not RT1-B antigens. After the (WF × BN)F_1 anti-LEW serum was absorbed with both LEW SE and LEW RBC, all cytotoxicity was removed, supporting the presence of only RT1-B (Ia) antigens in the subcellular membrane fragment preparation. Thus, several lines of evidence (Figs. 4–6) indicate that SE does not contain appreciable amounts of RT1-A antigen. Identical results were also obtained with Fm.

Attempts to adsorb BN SE to an immunoadsorbent column that contained anti-BN Ia antibodies were unsuccessful, as the bound material could not be recovered. The insoluble fragments were found to bind nonspecifically to Sepharose.

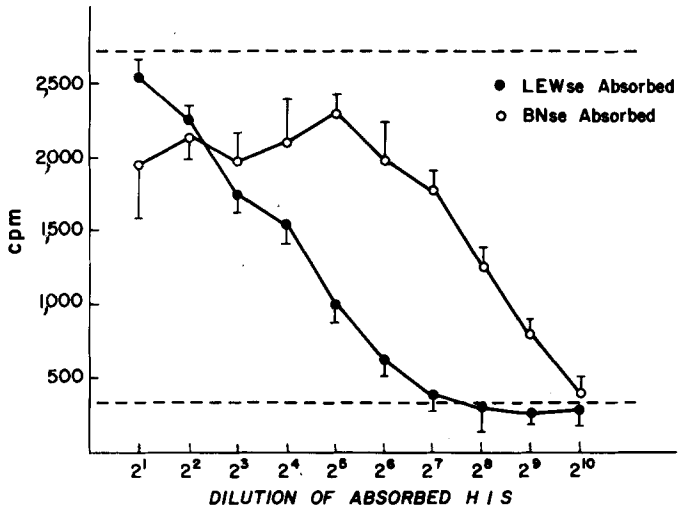

FIG. 5. CDC of (WF × BN)F_1 anti-LEW hyperimmune sera (HIS) absorbed with either LEW SE or BN SE on ^{51}Cr-labeled LEW Ad lymphocytes. Absorption with LEW SE resulted in an eightfold reduction in cytotoxicity as compared to HIS absorbed with BN SE. Results are expressed as counts per minute ± 1 SD of ^{51}Cr release.

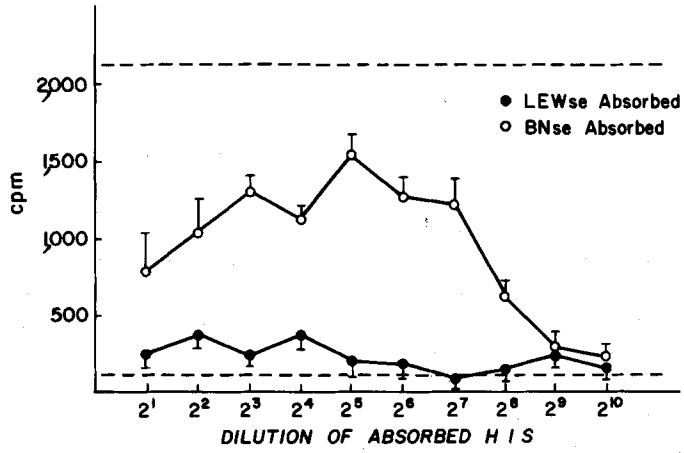

FIG. 6. CDC of LEW RBC absorbed (WF × BN)F_1 anti-LEW hyperimmune sera (HIS) absorbed with LEW SE or BN SE on ^{51}Cr-labeled LEW Ad lymphocytes. Absorption with LEW SE completely removes cytotoxicity, whereas absorption with BN SE does not change cytotoxicity.

Blocking of MLR Inhibition by Hyperimmune Antisera. LEW anti-BN sera inhibit the LEW anti-BN one-way MLR (14) as well as the Fm reponse (BN Fm plus LEW responder lymphocytes) (Table V). Prior absorption of hyperimmune sera with intact BN cells or BN Fm removes the antigen-specific MLR inhibiting effect. A second experiment is shown in Fig. 7. Three other experiments were similar. In all cases, absorption of LEW anti-BN hyperimmune serum with BN Fm removed the MLR blocking effect; although absorption with LEW Fm failed to do so.

Immunogenicity of Fm and SE. 10 LEW rats were immunized with BN Fm or SE.

TABLE V

Immunological Properties of Fm: Blocking of MLR Inhibition by Hyperimmune Serum after Absorption with Target Cell Fm

Responder cell*	Stimulator‡	Antiserum	cpm§
LEW NAd	—	—	1,834 ± 173
LEW NAd	—	LEW anti-BN	2,065 ± 130
LEW NAd	BN cells	—	36,324 ± 1,864
LEW NAd	BN cells	LEW anti-BN	1,298 ± 415
LEW NAd	BN cells	LEW anti-BN (abs with BN Fm)	29,446 ± 2,085
LEW NAd	BN Fm	—	20,614 ± 1,485
LEW NAd	BN Fm	LEW anti-BN	1,633 ± 147
LEW NAd	BN Fm	LEW anti-BN (abs with BN Fm)	22,618 ± 1,608
LEW NAd	BN Fm	LEW anti-BN (abs with LEW Fm)	2,047 ± 162
LEW NAd	BN cells	LEW anti-BN (abs with LEW Fm)	1,669 ± 751

* Cells used as stimulus are pretreated with mitomycin C.
‡ Whole cells treated with mitomycin C.
§ cpm, TdR incorporation at 72 h ± SEM.

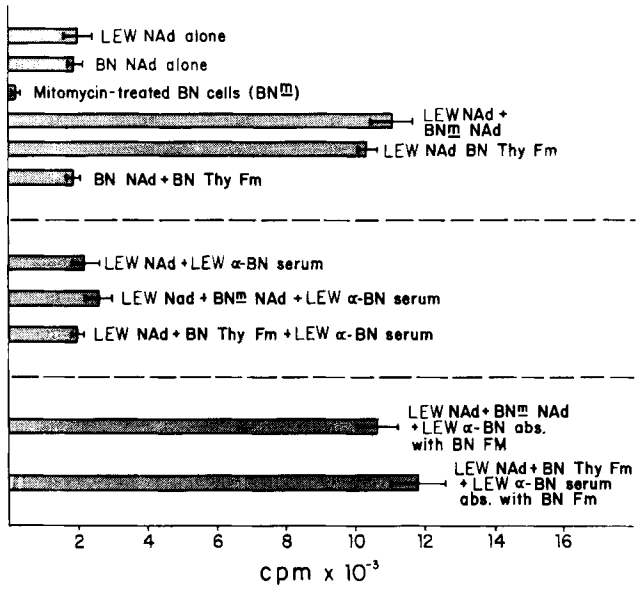

FIG. 7. Blocking of MLR inhibition by hyperimmune sera. LEW anti-BN hyperimmune sera inhibit both cell-cell one-way MLR reaction and the Fm MLR reaction, as shown in the top and middle sections. Prior absorption (abs.) with BN Fm removes this inhibition (lower section).

Significant and specific anti-BN Ia activity was detected in eight animals (Table VI) as assessed by the EA inhibition essay. The serums tested lacked classical anti-RT1-A activity, because hemagglutinating antibodies were not demonstrable. EAI was observed in neat sera, and in serial dilutions up to 1/32.

TABLE VI

Immunological Properties of Fm and SE: In Vivo Induction of Specific Anti-Ia Alloantisera in the Rat: LEW Rats Injected with BN Fm (Rats Fm-1 to FM-5) or BN SE (Rats SE-1 to SE-5)

Rat No.	BN lymphocytes		LEW lymphocytes	
	EA inhibition*	HA‡	EA inhibition	HA‡
	%		%	
Fm-1	33	2^1	0	2^0
Fm-2	31	2^0	0	2^0
Fm-3	25	2^0	0	2^0
Fm-4	6	2^0	0	2^0
Fm-5	47	2^1	3	2^0
SE-1	31	2^0	6	2^0
SE-2	39	2^0	0	2^0
SE-3	8	2^0	0	2^0
SE-4	47	2^1	0	2^0
SE-5	14	2^0	0	2^0

* EA inhibition data obtained with neat, undiluted sera. Significant EA inhibition was presented up to 1/32 dilution of sera from rats Fm-1, Fm-2, Fm-3, Fm-5, SE-1, SE-2, and SE-4.

‡ Hemagglutination titers. 2^1, positive with neat, undiluted sera.

Discussion

The rat MHC (RT1) resembles other mammalian systems that contain closely linked but separable regions as shown by laboratory recombination. Antisera reacting with classical Ag-B alloantigens of the RT1-A region are assayed via CDC and hemagglutination techniques. The MLR-stimulating antigens (RT1-B region) are detected by their ability to induce in vitro blastogenesis when whole cells are cultured with allogeneic lymphocytes (4). Other closely linked immune response genes control the ability of an animal to respond immunologically to various antigens. Mouse I-region associated (Ia) antigens are closely linked (or perhaps identical) to the specific immune response (Ir) gene products. They are primarily expressed on B lymphocytes, macrophages, epidermal cells, and endothelium; although virtually absent on peripheral T cells, platelets, and RBC (32). An apparently homologous antigenic system has been described in man and in the rat, and it is sometimes referred to as Ia-like or B-cell alloantigens (16, 19, 33–35). In the rat, these determinants are present on B cells, but are sparsely distributed upon T cells and platelets (14). Rat genes controlling Ia and Ir are all encoded by the RT1-B region. We have previously shown that a subpopulation of rat thymocytes are Ia+ and stimulate in MLR (19), and we now report that subcellular membrane fragments prepared from thymus cells (36) exhibit strain-specific stimulation of increased [^{3}H]TdR incorporation in unprimed allogeneic cells.

Two membrane fractions prepared by freeze-thaw lysis in hypotonic medium of thymocytes from three inbred rat strains (BN, BUF, and LEW) have both MLR-stimulating activity and absorb anti-Ia (RT1-B), but not anti-RT1-A antibodies. Fm is the fraction sedimentable between 800 and 60,000 *g*, whereas SE is sedimentable between 60,000 and 100,000 *g*. Virtually all of the RT1-A antigenicity is present in the first crude sediment (800 *g*). Failure of other investigators to obtain MLR-like effects from membrane preparation may be the result of nonspecific suppressor activity present in lysates. We found, for example, that Fm and Se prepared from Ad

spleen cells failed to produce an MLR-like effect. In fact, spleen Fm depressed in vitro [^{3}H]TdR incorporation in both allogeneic and syngeneic lymphocytes. Thymus Fm and SE also showed loss of stimulatory activity at high concentrations. Morphological studies (eosin dye exclusion) demonstrated that the suppression of [^{3}H]TdR incorporation by splenic Fm and SE was not a result of cytotoxic effects of either preparation.

Incorporation of [^{3}H]TdR in cells confronting allogeneic membrane fragments is not proof in itself of a clonally-directed response to MHC antigens. Therefore, two additional sets of experiments were undertaken to show MHC specificity. In the first, TdR suicide of WF cells (RT1^u) responding to LEW (RT1^l) Fm or SE provided evidence that WF responder cells included all clones with receptors for LEW but not BN (RT1^u) (Table III). The identity of the proliferative response to SE or Fm with that induced by cell-stimulated MLR is clear. Suicide with high specific activity [^{3}H]TdR after primary stimulation with whole cells, Fm or SE results in specific clonal deletion of the same cells, leaving response to another haplotype intact. Second, we demonstrated that responsiveness to BN Fm and SE segregated with the MLR response to parenteral typing cells in the F_2 progeny of LEW and BN crosses. (Table IV) When a group of (LEW × BN)F_2 animals were analyzed for MHC antigens by hemagglutination, B-cell CDC, and MLR typing, four animals typed as *n/n*, nine as *l/n*, and six as *l/l*. Study of the response of lymph node cells from these animals in culture with BN(*n/n*) subcellular fragments showed that the MLR response to both intact cells and these subcellular antigens were concordant and linked to the MHC. Hence, it is extremely unlikely that the major determinants stimulating proliferation in a strain-specific pattern are other than the RT1-B MLR determinants. Further specificity is shown by the failure of LEW and F344 fragments to stimulate each other's cells, even though they differ by a non-MLR-related Ia antigen (Ia-2) of RT1. (Table II).

One feature of SE stimulation, presently unexplained, deserves mention. Not only do allogeneic cells exposed to SE respond more rapidly than those exposed to Fm or intact cells, but the SE system does not require the presence of phagocytic cells. (Fig. 3) The exact role for phagocytic cells, which can be of either stimulator or responder phenotype, in the MLR is uncertain. Perhaps some form of antigen processing is required before the T cell response begins, whereas SE already presents antigen in an altered form. It is also possible that the thymic SE contains additional stimulatory factor(s).

Absorption of hyperimmune sera with subcellular antigens results in removal of activity against RT1-B but not RT1-A antigens. Dickler and Sachs (30) have demonstrated that anti-Ia sera but not anti-K or anti-D antibodies raised in congenic mouse lines specifically blocked Fc receptor binding of aggregated IgG, while antibodies directed against the K and D regions of the MHC were ineffective. Soulillou et al. (14) and Catto et al. (15), using EA rosette formation as an assay of Fc receptor function, showed that rat alloimmune serum, after platelet absorption to remove the anti-RT1-A or AgB antibodies, also blocked EA rosette formation by target lymphocytes, and that immunoabsorbent-purified anti-RT1-A did not block EA rosettes. Parallel results have been reported in man by Solheim et al. (34) and Suthanthiran et al. (33). Absorption of rat hyperimmune LEW anti-BN alloantisera with target-strain lymphocytes removes their EA-rosette-inhibiting properties and their hemagglutinating properties as well. In contrast, the absorption of these antisera with either

Fm or SE preparations removes their EA rosette-inhibiting activity, but does not alter the hemagglutinating effect. (Fig. 4) Therefore, both Fm and SE display strain-specific Ia antigenicity but are virtually lacking in RT1-A (Ag-B) antigens. Shown in Figs. 5 and 6 is the sequential absorption of a (WF × BN)F_1 anti-LEW serum with LEW SE and LEW RBC, tested by complement-dependent lymphocytotoxicity. In the first stage, absorption with SE removed all RT1-B activity; however, residual activity against both B and T cells remained. The latter (anti-RT1-A) was easily absorbed with LEW RBC, and indicates the relative lack of RT1-A antigens in the SE. Hence, application of different techniques for detection of RT1-A antibodies (hemagglutination and CDC before and after RBC absorption) and for RT1-B (Ia) antibodies (EAI and CDC upon purified B lymphocytes) show that these membrane-preparations are virtually devoid of RT1-A antigens. This fact is further supported by in vivo immunizations, because injection of allogeneic Fm or SE into recipient animals stimulated the production of anti-Ia antibodies but not anti-RT1-A antibodies (Table VI). Preliminary studies with human peripheral blood cells show that Fm and SE can also stimulate allogeneic cells, and contain HLA-DR, but not HLA-A, B, and C antigens (37, 38).

Our data also bear on the argument that restimulation of primed cells with cell membranes or solubilized membrane proteins to elicit cytotoxic T cell differentiation (6–10) does not reflect specific restimulation of memory cells by antigen, but may result from nonspecific help coming from Ia-primed helper cells (39). Because we now show that membrane fragments can, in fact, elicit primary allogeneic responses in vitro, experiments with fragments should not be viewed as measuring restimulation of primed cells only. The use of Fm or SE, devoid as they are of β_2-microglobulin-containing antigens (class I), should be useful probes for provision of either primary or secondary Ia-related helper signals in studies on the specificity of effector-cell generation.

Summary

Rat thymocyte membrane fractions have been prepared which exhibit strain-specific primary mixed-lymphocyte reaction (MLR)-stimulating and Ia (RT1-B) antigenic properties. These preparations lack the antigenicity of classical, serologically-defined RT1-A (Ag-B) antigens, as defined by in vitro serologic assays. Furthermore, after immunization of allogeneic hosts, specific anti-Ia and MLR-blocking antibodies, but not anti-AgB, alloantibodies are elaborated. Thymidine suicide experiments show that the same clones respond to whole cells and the fragments made from those cells, and the response segregates appropriately in F_2 progeny as a major histocompatibility complex (RT1)-linked phenomenon. Hence, it is possible to generate Ia-related allogeneic helper signals in primary, as well as secondary, in vitro responses, using subcellular membrane fragments that have restricted expression of RT1-B-, but not RT1-A-, encoded antigens.

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