

## CELL-MEDIATED CYTOTOXICITY DURING REJECTION AND ENHANCEMENT OF ALLOGENEIC SKIN GRAFTS IN RATS\*

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Measurement of cell-mediated cytotoxicity (CMC)<sup>1</sup> directed to cell surface antigens has been facilitated by the elaboration of a relatively simple reproducible test in which <sup>51</sup>Cr is released from labeled target cells (1-5). Other investigators have examined certain facets of the test (6-18) and have applied the technique to studies of tumor allograft systems (3-5, 16, 19, 20) and soluble antigens bound to the surface membrane of target cells (18, 21-26).

The purpose of this study was to detect the appearance of cytotoxic lymphocytes (CL) in spleens and draining lymph nodes of rats grafted with allogeneic skin by means of a modified <sup>51</sup>Cr release assay and to quantitate CMC during rejection and prolonged survival of allografts induced by enhancing antibodies. With <sup>51</sup>Cr-labeled embryonic fibroblasts (EFB) as target cells derived from donor strain rats that were the source of immunizing skin grafts, and with cytotoxic lymphoid cells from sensitized recipients, the level of cellular immunity could be determined in recipients of skin allografts.

### Materials and Methods

*Animals.*—150-200-g adult and neonatal Lewis (Le) rats were both used as skin-graft recipients and donors of aggressor cells for assays of CMC in vitro. Brown-Norway (BN) rats were a source of skin allografts and of target cells for CMC assays. Both inbred strains were obtained from our own breeding colony or from a commercial breeder (Simonsen Laboratories, Gilroy, Calif.). BN skin grafts, 1.5 cm on edge, were applied to the nuchal region of 7-8-day-old Le neonates or to the axillae of adult Le rats. The latter were either normal, injected intraperitoneally with 5 mg of cyclophosphamide<sup>2</sup> daily beginning 4 days before grafting, or neonatally thymectomized 24-48 hr after birth and used 100 or more days later. Completeness of

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<sup>1</sup> *Abbreviations used in this paper:* BN, Brown-Norway; CL, cytotoxic lymphocytes; CMC, cell-mediated cytotoxicity; EAS, enhancing antiserum; EFB, embryonic fibroblasts; HARTD, horse antiserum to rat thoracic duct cells; EIGG-<sup>125</sup>I, enriched, labeled IgG with enhancing activity; LABN, Lewis anti-Brown-Norway; Le, Lewis; MEM, minimal essential medium; NLS, normal Lewis serum; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells.

<sup>2</sup> Generously supplied by Mead Johnson & Co., Evansville, Ind.

thymectomy was ascertained at the end of experiments by histologic examination of the tissue in the operative site.

*Antisera and Antibodies.*—Enhancing antisera (EAS) were prepared in Le adult rats that were grafted with skin two times and infused with spleen cells two times from BN donors. Complete details of preparation are presented in references 27 and 28. 0.3 ml of Le anti-BN (LABN) EAS or of normal Le serum (NLS) were injected intraperitoneally into experimental and control neonates, respectively, on the day of grafting.

An IgG fraction was prepared from EAS; and for some experiments it was labeled with  $^{125}\text{I}$  and enriched by adsorption to and elution from BN lymphocytes, as described previously (29). The enriched, labeled IgG (EIgG- $^{125}\text{I}$ ) with enhancing activity showed a specific binding of 38% to BN spleen cells and 19% to BN embryonic fibroblasts (BN-EFB).

Horse antiserum to rat thoracic duct cells (HARTD)<sup>3</sup> was precipitated two times with 50%  $(\text{NH}_4)_2\text{SO}_4$ . The redissolved precipitate was passed over a diethylaminoethyl (DEAE)-50 Sephadex column (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) equilibrated with phosphate-buffered saline (PBS). The IgG was eluted, concentrated, and divided into two equal portions. Both were absorbed with rat erythrocytes and with NLS insolubilized by 2.5% glutaraldehyde (30). One portion was further absorbed four times with  $5 \times 10^9$  Le thymocytes and the other once with  $10^9$  Le bone marrow cells. HARTD IgG, absorbed with thymocytes, did not release any  $^{51}\text{Cr}$  from labeled BN-EFB. HARTD IgG absorbed with bone marrow cells released 50% of the isotope at a dilution of 1:16.

*Cytotoxic Lymphocytes (CL) and Target Cells.*—Spleens and draining lymph nodes were removed aseptically from Le rats 3–13 days after placement of BN skin grafts or after subcutaneous inoculation of 0.2 ml of incomplete Freund's adjuvant (control rats). Cell suspensions were prepared in Eagle's minimal essential medium (MEM), washed two times, and adjusted to a concentration of  $2 \times 10^7$  cells per milliliter.

Peripheral blood lymphocytes were collected from 10–12 ml of heparinized blood, as follows. The blood was incubated for 60 min at 37°C in sterile tissue culture flasks. An equal volume of 3% Dextran 250 (Pharmacia Fine Chemicals, Inc.) in PBS was then added to the blood, and the mixture was incubated in plastic cylinders at 37°C for an additional 30 min. The supernatant was removed, mixed with cold MEM, and centrifuged for 15 min at 1500 rpm. Red blood cells in the pellet were lysed with 0.83% ammonium chloride, which was removed by three washes of MEM. Mononuclear cells ranged from 5 to  $10 \times 10^7$  cells per 10–12 ml of original blood sample, and were 96–98% of the total cell count.

BN-EFB and Le-EFB were grown as monolayers in plastic tissue culture flasks from 12–14-day-old embryos that were minced and trypsinized. For use in CMC assays, the monolayers were flooded with 7.5 ml of trypsin ethylenediaminetetraacetate (EDTA) solution (0.5 and 0.2 mg/ml, respectively) for 10 min. The suspended cells were washed two times in MEM, and  $2 \times 10^7$  cells were labeled with 200  $\mu\text{Ci}$  of  $\text{Na}_2^{51}\text{CrO}_4$  by incubation for 30 min at 37°C in 5%  $\text{CO}_2$  and 95% air. Suspensions were washed again four times and adjusted so that 50  $\mu\text{l}$  contained  $10^5$  cells (20,000–25,000 cpm). 50  $\mu\text{l}$  of EFB were used as target cells.

In one experiment, BN peritoneal exudate cells (BN-PEC) were used. They were flushed from the peritoneal cavity 3 days after intraperitoneal injection of 8 ml of sterile 10% proteose peptone, washed three times in MEM, and adjusted to a concentration of  $2 \times 10^7$  cells/ml. They were labeled with 400–500  $\mu\text{Ci}$  of  $\text{Na}_2^{51}\text{CrO}_4$  as described above. 50  $\mu\text{l}$  contained  $10^5$  cells (20,000 cpm) and were used as target cells.

*Cell-Mediated Cytotoxicity (CMC) Assay In Vitro.*—The method of Brunner et al. (3) was slightly modified.  $10^5$  target cells were incubated with  $10^7$  aggressor lymphoid cells in  $12 \times 75$ -

<sup>3</sup> A gift from Upjohn Co. Kalamazoo, Mich.

<sup>4</sup> International Chemical and Nuclear Corp., Irvine, Calif. Specific activity, 400 mCi/mg Cr.

mm round-bottom glass or plastic tissue culture tubes. Volume was adjusted to 1 ml with supplemented MEM.<sup>5</sup> The tubes were incubated for 2, 6, and 7.5 hr at 37°C in 5% CO<sub>2</sub> and 95% air on a rocking platform (8 tilts per min). Optimal incubation period was 7.5 hr, and all data reported here are for this span of time unless otherwise noted. After incubation, 1 ml of MEM was added to each tube, tubes were centrifuged for 15 min at 1200 rpm, and 1 ml of supernatant was removed and counted for radioactivity. Maximal <sup>51</sup>Cr release was obtained by lysis of target cells with 5% NP40<sup>6</sup> and was 87.9 ± 2.5% (SD) of <sup>51</sup>Cr input. Each test was run in four or five replicate samples, and <sup>51</sup>Cr release is recorded as the mean of pooled cells from 2 to 3 control and experimental animals or from 3 to 10 individual graft recipients for the kinetic studies.

$$\text{Specific } ^{51}\text{Cr release} = \frac{^{51}\text{Cr release with lymphoid cells} - \text{spontaneous } ^{51}\text{Cr release}}{^{51}\text{Cr release with 5\% NP40} - \text{spontaneous } ^{51}\text{Cr release}} \times 100.$$

Mean spontaneous <sup>51</sup>Cr release at 7.5 hr of incubation was 10.9 ± 1.1% (SD) of <sup>51</sup>Cr input (26 trials with BN-EFB) and 15.8 ± 1.8% (6 trials with BN-PEC). Tests in which the difference between maximal <sup>51</sup>Cr release minus spontaneous <sup>51</sup>Cr release was less than 70% were discarded.

In some experiments assays were carried out after preincubation of target cells with antibodies. To each of five replicas, serial dilutions of heat-inactivated NLS, EAS, LABN-IgG (0.57 mg), or EIgG-<sup>125</sup>I (10 μg), in 0.1-ml volumes of MEM, were added 10<sup>5</sup> <sup>51</sup>Cr BN-EFB cells. The mixtures were incubated for 30 min at 37°C. Immune or nonimmune aggressor cells were then added to each test tube, and volume was adjusted to 1 ml. Incubation was continued for 7.5 hr. In another study, 5 × 10<sup>7</sup> LABN aggressor cells were incubated in the following preparations for 45 min at 37°C: 0.5 ml NLS; 0.5 ml LABN EAS; 0.5 ml EIgG-<sup>125</sup>I (120 ng); 0.5 ml HARTD IgG absorbed with bone marrow or thymus cells (14 mg). Aggressor cells were washed two times in MEM, and 10<sup>7</sup> cells were distributed to each of five test tubes containing 10<sup>5</sup> <sup>51</sup>Cr BN-EFB target cells. Incubation was continued for 7.5 hr. When complement was used, it was added as fresh guinea pig serum diluted 1:2 in MEM.

## RESULTS

*CMC in Adult Le Rats with BN Skin Allografts.*—A first series of experiments was designed to compare the effect of different kinds of grafting on the induction and expression of CMC in adult Le rats (Fig. 1). The most vigorous CMC was elicited by first-set BN skin and kidney grafts. Intravenous injections of allogeneic spleen cells failed to produce, 7 days later, significant CMC. Spleens from hyperimmune Le rats that were immunized with two BN skin grafts and two intravenous BN spleen cell infusions at weekly intervals exhibited less CMC 8 days after the last immunizing procedure than did spleens of Le rats immunized with first-set BN skin grafts.

Specificity of CMC for sensitizing AgB antigens was established by demonstrating that LBNF<sub>1</sub> spleen cells, removed 7 days after a BN skin graft, were not cytotoxic to BN-PEC (Fig. 1). Similarly, LABN aggressor cells were unable to destroy syngeneic Le-EFB (Fig. 2). Also, no significant release of

<sup>5</sup> Contains 10% heat-inactivated fetal calf serum; 4× nonessential amino acids; 1× glutamine; 100 units penicillin/ml; 2.5 μg fungizone/ml; 100 μg streptomycin/ml; MgCl<sub>2</sub> × 6H<sub>2</sub>O, 0.002 mM/ml; CaCl<sub>2</sub>, 0.0007 mM/ml.

<sup>6</sup> Generously supplied by Shell Chemical Co., New York.

$^{51}\text{Cr}$  was observed when LABN aggressor cells were mixed with labeled Le-EFB and unlabeled BN-EFB. Thus, in this assay, a nonspecific toxic agent, generated by the interaction of immune aggressor cells with specific AgB antigens, was not detected. More likely lysis of target cells was effected by a direct cell to cell reaction. Specific  $^{51}\text{Cr}$  release increased with increasing ratios of aggressor to target cells over a range of 10:1 to 200:1 (Fig. 3). Beyond a ratio of 200:1,  $^{51}\text{Cr}$  release from target cells decreased.

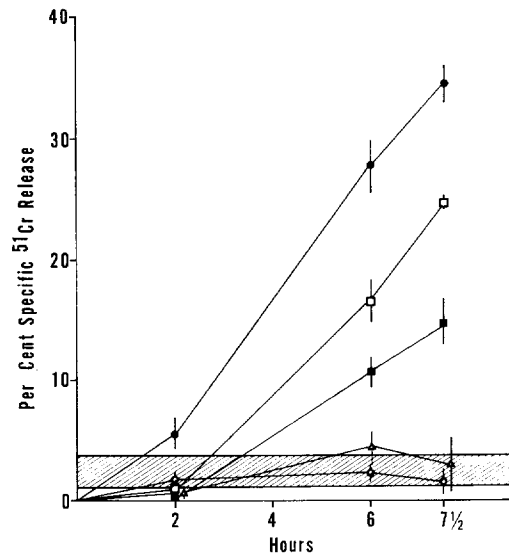


FIG. 1. CMC after different immunization procedures. Each point shows the specific  $^{51}\text{Cr}$  release from  $10^5$  BN-PEC incubated with  $10^7$  Le or LBNF<sub>1</sub> spleen cells and represents the mean of four replicate samples of pooled cells from three rats,  $\pm 1$  SD (vertical lines). Symbols: ●, first-set BN skin graft, 8 days before; □, first-set BN kidney graft, 5 days before; ■, two BN skin grafts, two BN spleen cell infusions, each 1 wk apart, 8 days after last injection; ○, first-set BN skin graft on LBNF<sub>1</sub> recipients, 8 days before; △, 1 i. v. infusion of  $2 \times 10^8$  BN spleen cells, 8 days before. Shaded area: mean specific  $^{51}\text{Cr}$  release  $\pm 1$  SD by normal Le spleen cells.

A kinetic study of CMC in the spleens and lymph nodes of grafted Le adult females was carried out (Fig. 4). CMC was first detected 5 days after grafting, in draining lymph nodes of graft recipients, and 1 day later in spleens of similarly prepared rats. Peak activity was constantly observed at 7 or 8 days, when rejection of the allogeneic skin was near completion. (Median survival time in these recipients was  $7.5 \pm 0.7$  days.) CMC dropped quickly thereafter and reached background levels in the lymph nodes on day 9, and in spleens on days 10 and 11.

CMC in peripheral blood was also determined in a second series of Le adult females grafted with BN skin (Fig. 5). CL were first detected on day 7, and

peaked on day 8, with a rapid return to background levels. The curve depicted in Fig. 5 is similar to that of Fig. 4 and suggests that the rise and fall of CL in both the peripheral blood and the lymphoid tissues are temporally related.

*CMC in Allografted Le Neonates, Cyclophosphamide-Treated, and Neonatally Thymectomized Le Adult Rats.*—Previous studies have demonstrated that in Le neonates and in cyclophosphamide-treated or neonatally thymectomized adult

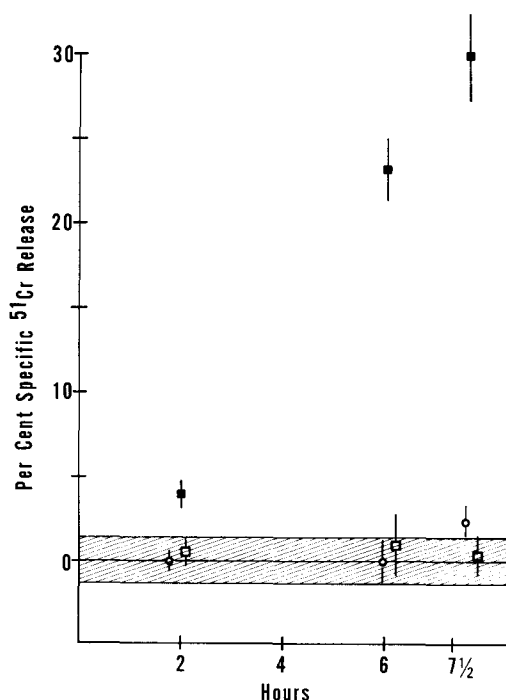


FIG. 2. Specificity of CMC. Each point shows the specific  $^{51}\text{Cr}$  release from  $10^5$  BN-EFB by  $10^7$  LABN aggressor cells 7 days after placement of BN skin grafts and represents the mean of five replicate samples of pooled cells from three rats,  $\pm 1$  SD (vertical lines). Symbols:  $\square$ , BN-EFB;  $\blacksquare$ , Le-EFB;  $\circ$ ,  $^{51}\text{Cr}$  Le-EFB + BN-EFB. Shaded area: mean  $^{51}\text{Cr}$  release  $\pm 1$  SD from  $^{51}\text{Cr}$  BN-EFB and  $^{51}\text{Cr}$  Le-EFB incubated 7 1/2 hr without aggressor cells.

rats survival of skin allografts may be significantly prolonged by administration of EAS (29). It was of interest, therefore, to investigate CMC in lymphoid tissues of rats manipulated in this fashion. 7 days after placement of a BN skin graft, recipients of all three groups showed significantly less CMC than normal adult rats (Table I). Specific  $^{51}\text{Cr}$  release by aggressor spleen cells of neonatally thymectomized or cyclophosphamide-treated recipients did not exceed the release caused by nonimmune spleen cells. Aggressor cells from 15-day-old neonates and from incompletely thymectomized graft recipients re-

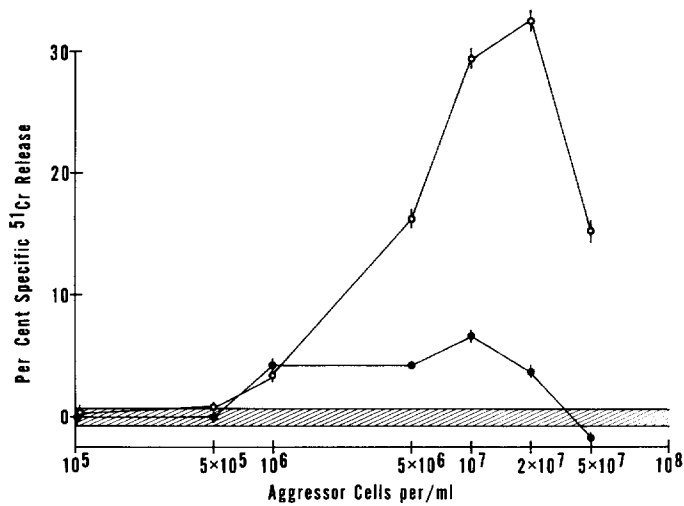


FIG. 3. Effect of varying numbers of aggressor cells on specific  $^{51}\text{Cr}$  release. Each point shows the specific  $^{51}\text{Cr}$  release from  $10^5$  BN-EFB incubated for 7 1/2 hr with increasing numbers of aggressor cells and represents mean of six replicate samples of pooled cells from three rats,  $\pm 1$  SD (vertical lines). Symbols:  $\circ$ , Le spleen cells from allografted donors;  $\bullet$ , Le non-immune spleen cells. Shaded area:  $^{51}\text{Cr}$  release  $\pm 1$  SD from target cells (BN-EFB) without aggressor cells.

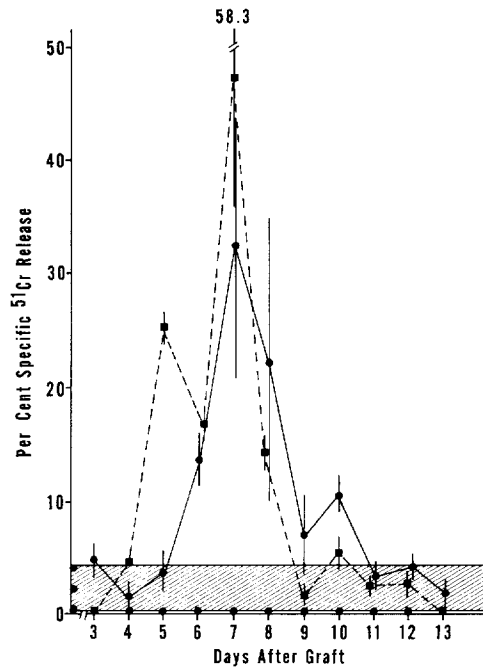


FIG. 4. Kinetic study of CMC in spleens and lymph nodes of allografted Le rats. Each point shows the specific  $^{51}\text{Cr}$  release from  $10^5$  BN-EFB incubated for 7 1/2 hr with  $10^7$  Le spleen and lymph node cells and represents the mean of four replicate samples from each of 3 to 10 rats,  $\pm 1$  SD (vertical lines). Donors of aggressor cells were grafted with BN skin. Symbols:  $\circ$ — $\circ$ , Le-sensitized spleen cells;  $\square$ — $\square$ , Le-sensitized lymph node cells. Shaded area: Mean specific  $^{51}\text{Cr}$  release  $\pm 1$  SD from  $10^5$  BN-EFB incubated with  $10^7$  Le spleen or lymph node cells from rats inoculated with incomplete Freund's adjuvant (28 animals).

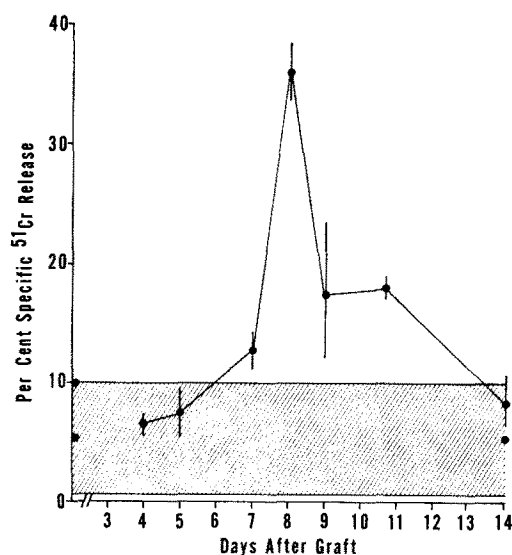


FIG. 5. CMC in peripheral blood of allografted adult Le rats. Each point shows the specific <sup>51</sup>Cr release from 10<sup>5</sup> BN-EFB and represents the mean of five replicate samples of pooled cells from two or three animals, ± 1 SD (vertical lines). Symbols: ○—○, 10<sup>7</sup> cells from allografted rats. Shaded area: mean specific <sup>51</sup>Cr release ± 1 SD from 10<sup>5</sup> BN-EFB and 10<sup>7</sup> nonimmune lymphoid cells (13 animals).

TABLE I  
Specific <sup>51</sup>Cr Release from BN-EFB\* Incubated with Le Lymphoid Cells

Source of Le cells	% specific <sup>51</sup> Cr release † ± SD		Total cells per spleen × 10 <sup>8</sup>	CL per spleen § × 10 <sup>3</sup>
	Le spleen cells	Le lymph node cells		
Normal adult rats (21)	5.0 ± 3.6	n.d. ¶	4.0	0
Adult allografted rats (10)	32.2 ± 11.4	47.1 ± 11.2	3.0–5.0	1088
15-day-old allografted rats (3)	18.6 ± 1.1	—**	0.8–1.2	136
Cyclophosphamide-treated allografted adult rats (3)	8.8 ± 2.7	0.7	3.0	114
Neonatally thymectomized allografted adult rats (5)				
Complete	8.0 ± 1.1	—**	4.0	93
Incomplete	15.6 ± 3.8	—**	3.1	424

\* Embryonic fibroblasts.

† See Materials and Methods for definition.

§ Calculated cytotoxic lymphocytes (CL) after correction for CL in normal Le spleen. Animals were compared 7 days after BN skin grafts.

|| Figures in parentheses indicate number of rats.

¶ Not done.

\*\* Lymph node cells pooled with spleen cells.

leased about 50% of the  $^{51}\text{Cr}$  released by aggressor cells of untreated allografted adult rats.

Based on the postulate that one aggressor cell kills one target cell, i.e. the one-hit theory (31, 32), the number of CL per spleen was calculated. In adult unmanipulated Le recipients of allografts, CL were 0.5% of lymph node cell suspensions and 0.3% of suspensions of spleen cell and peripheral blood lymphocytes tested at the height of CMC. In neonates, cyclophosphamide-treated and neonatally thymectomized adults, CL per spleen were calculated to be 10% of these values (Table I).

*Effect of EAS on Allografted Le Neonates.*—Litters of eight Le neonates were grafted on day 7 with BN skin. On the day of grafting, four cubs were injected

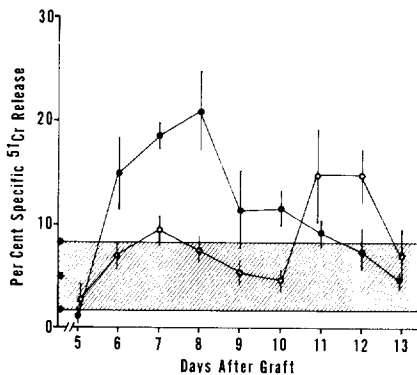


FIG. 6. Effect of EAS on CMC in allografted Le neonates. Each point shows the specific  $^{51}\text{Cr}$  release from  $10^5$  BN-EFB and represents five replicate samples of cells from each of 3 to 10 rats,  $\pm 1$  SD (vertical lines). Donors of aggressor cells were grafted with BN skin 7 days previously. Symbols: ●, NLS (normal Le serum); ○, EAS (enhancing antiserum). Shaded area: mean specific  $^{51}\text{Cr}$  release  $\pm 1$  SD from BN-EFB incubated with cells from normal Le rats (21 animals).

intraperitoneally with 0.3 ml of EAS and four with 0.3 ml of NLS. From 5 to 13 days later spleens and axillary lymph nodes of each animal were pooled and assayed for CMC (Fig. 6). Peak CMC in spleens of neonates injected with NLS occurred 7 and 8 days after grafting, at the same time as it was observed to occur in allografted unmanipulated adult rats. By contrast, peak CMC in spleens of neonates injected with EAS occurred on days 11 and 12, 3–4 days later than peak activity in neonates given NLS. The level of CMC in Le neonates, as measured by specific  $^{51}\text{Cr}$  release, was only about 45% of that calculated in adult spleens. At day 7 the level of CMC in EAS-treated neonates was reduced to about 50% of the CMC found in neonates treated with NLS, and to about 20–30% of the CMC calculated in lymphoid tissues of unmanipulated adults (Figs. 4 and 6).

*Blockade of CMC In Vitro by Alloantibody and by HARTD Antibodies.*—



When  $^{51}\text{Cr}$  BN-EFB were preincubated in LABN-EAS, LABN-IgG, and EIgG- $^{125}\text{I}$ , activity of aggressor cells was blocked (Fig. 7 *a*). Blocking was incomplete, and CMC was reduced to about 50% of the CMC observed with preincubation of NLS. As EAS was diluted, blocking was less effective, until no significant effect of EAS was noted when it was diluted 1:16.

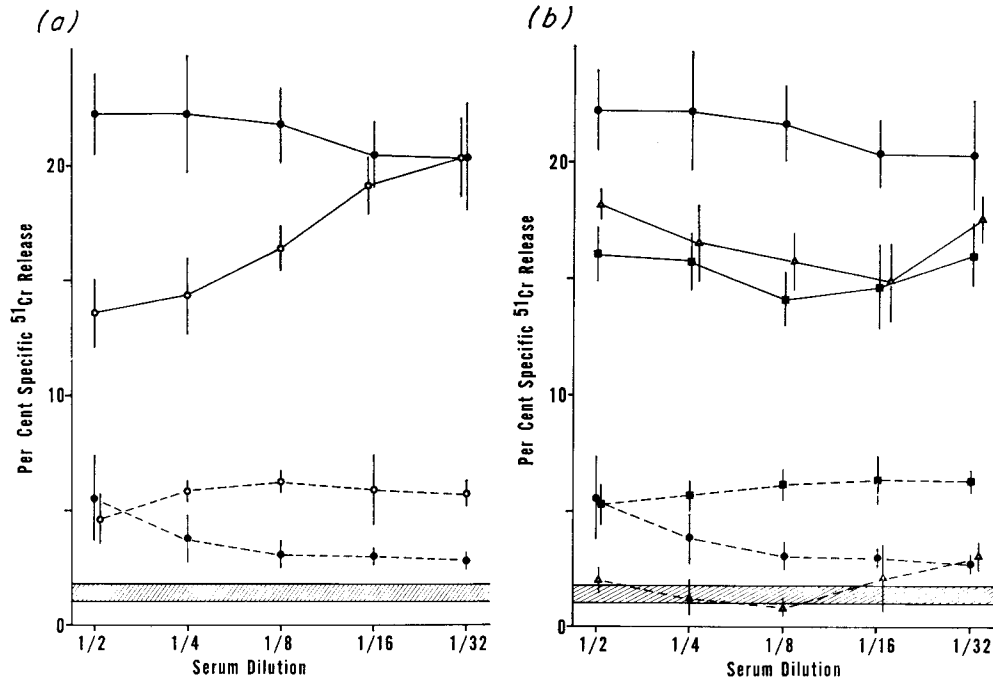


FIG. 7. Effect in vitro of blocking antibodies on  $^{51}\text{Cr}$  release from target cells incubated with blocking antibodies. Each point shows specific  $^{51}\text{Cr}$  release from  $10^5$  BN-EFB and represents the mean of five replicate samples,  $\pm 1$  sd (vertical lines). BN-EFB target cells were incubated with normal serum or blocking antibodies for 45 min before addition of  $10^7$  aggressor cells. (a) Symbols:  $\bullet$ —, BN-EFB + NLS + Le CL;  $\circ$ —, BN-EFB + EAS + Le CL;  $\bullet$ ---, BN-EFB + NLS + nonimmune Le cells;  $\circ$ ---, BN-EFB + EAS + nonimmune Le cells. Shaded area: mean specific  $^{51}\text{Cr}$  release  $\pm 1$  sd from BN-EFB and nonimmune lymphoid cells, without serum or antibodies. (b) Symbols:  $\bullet$ —, BN-EFB + NLS + CL;  $\Delta$ —, BN-EFB + EIgG- $^{125}\text{I}$  + CL;  $\blacksquare$ —, BN-EFB + EAS IgG + CL;  $\bullet$ ---, BN-EFB + NLS + nonimmune Le cells;  $\Delta$ ---, BN-EFB + EIgG- $^{125}\text{I}$  + nonimmune Le cells;  $\blacksquare$ ---, BN-EFB + EAS IgG + nonimmune Le cells. Shaded area: same as above.

Lymphocyte suspensions derived from nongrafted control rats exhibited slight specific  $^{51}\text{Cr}$  release, about 6% when a 1:2 dilution of NLS was incubated with target cells and 3% when higher dilutions of NLS were used. When EAS was preincubated with target BN-EFB and, later, when lymphoid cells from nonimmune rats were added, there was a specific release of  $^{51}\text{Cr}$  up to 7%; and this was statistically significantly greater than the release observed in control

incubations (Fig. 7 *a*). This specific release of  $^{51}\text{Cr}$  with EAS and normal lymphoid cells was consistently observed in five separate trials. Similar results were observed when target cells were preincubated with LABN-IgG, but not with EIgG- $^{125}\text{I}$  (Fig. 7 *b*).

Preincubation of LABN aggressor cells in EAS, in LABN-IgG, and in

TABLE II  
*Effect of NLS,\* EAS, and EIgG- $^{125}\text{I}$  on Specific Release of  $^{51}\text{Cr}$  by LABN Aggressor Cells*

Preincubation† with	% specific $^{51}\text{Cr}$ release $\pm$ SD
MEM	49.7 $\pm$ 2.1
0.5 ml NLS	47.7 $\pm$ 1.5
0.5 ml EAS	46.6 $\pm$ 1.6
0.5 ml (60 ng) EIgG- $^{125}\text{I}$	50.9 $\pm$ 2.5

\* NLS = normal Le serum; EAS = enhancing antiserum; EIgG- $^{125}\text{I}$  = enriched labeled IgG with enhancing activity; LABN = Lewis anti-BN.

†  $5 \times 10^7$  lymphoid cells from allografted Le recipients were incubated for 30 min, at 37°C, and washed two times.  $10^7$  cells, in five assays for each group, were mixed with  $10^5$  BN-EFB.

TABLE III  
*Effect of HARTD on Specific Release of  $^{51}\text{Cr}$  by LABN Aggressor Cells*

Preincubation* with	% inhibition of specific $^{51}\text{Cr}$ release	% dead cells†
HARTD§	66.1	24
HARTD§ + C	96.7	91
HARTD absorbed¶	0.0	18
HARTD absorbed + C	5.0	16
C	6.9	19

\*  $10^7$  lymphoid cells from allografted Le rats were incubated for 45 min, at 37°C, and washed two times before mixing with  $10^5$  BN-EFB.

† As determined by staining with trypan blue.

§ Horse antiserum to rat thoracic duct cells, IgG fraction, absorbed once with  $1 \times 10^9$  rat bone marrow cells.

|| Complement (C) used as fresh guinea pig serum diluted 1:2 with MEM.

¶ Absorbed four times with  $5 \times 10^9$  rat thymus cells.

EIgG- $^{125}\text{I}$  did not block CMC in vitro (Table II), nor did NLS display any blocking activity under these conditions.

By contrast, when HARTD IgG, absorbed with rat bone marrow cells, was first incubated with aggressor cells and then washed out before mixing aggressor and target cells, inhibition of CMC was significant (Table III). In the presence of complement, the same antibody preparation apparently killed aggressor cells, and CMC was completely inhibited. If HARTD IgG was absorbed with thymocytes, its inhibiting action against aggressor cells was removed.

## DISCUSSION

The data gathered in this report support three broad conclusions. First, CMC can be measured quantitatively in the lymphoid tissues and peripheral blood of allografted adult rats, and displays a response that resembles the rise and fall of antibody on a first exposure to antigen. CMC was first detected in draining lymph nodes 5 days after grafting, peaked 2-3 days later, and promptly returned to background levels by day 9. Its appearance preceded any visible gross changes in the skin allograft and coincided with the earliest mononuclear cell infiltrations as determined by histologic examination (33). A period of 5 days was needed for CMC to be detectable, and presumably this time interval allowed for processing of alloantigens, recognition by antigen-reactive cells, conversion to effector elements, and accumulation of sufficient numbers to be assayed. The rapid decline of CMC in lymphoid tissues of the grafted rat might have been the result of several events, e.g., the convention of CL and their destruction in the dying graft, the expulsion of histoincompatible AgB antigens from the host, and the subsequent absence of AgB antigens, which no longer drove the cellular immune response to recruit and/or reproduce additional effector elements. The prolonged persistence of CMC, described by Brunner et al. (4), may reflect the difference between a proliferating neoplastic graft and a normal tissue graft.

The kinetics of CMC in the lymphoid tissues and peripheral blood were similar. Unfortunately, measurements of CMC in lymphoid tissues and in peripheral blood were made in two different groups of rats. It was not possible, therefore, to relate precisely the temporal development of CMC in these two anatomic sites, or to state if CL migrated from lymphoid tissues into the blood, or if CL were activated peripherally and congregated in lymphoid tissues. Nor was it possible to ascertain if the CL in the peripheral blood were related to circulating activation lymphocytes described by Hersh et al. (34).

The CMC assayed in this report belonged to the category first described by Snell et al. (35) a decade ago, and later characterized more carefully by Wilson et al. (36) and Brunner and his colleagues (3-5, 14, 16). These latter workers demonstrated a direct killing action by thymus-derived cells against target cells with histoincompatible antigens. Both conventional-type antibody and complement did not play any role in the cytotoxic activities described. Our observations with rat cells coincided with their findings for mouse systems. Neither alloantibody nor complement contributed to the killing action of CL in our assay. Furthermore, on a semilogarithmic scale, a nearly straight-line dose-response curve depicted the action of an increasing number of aggressor cells on a fixed number of target cells over a wide range, and no toxic factor was detected in the medium after interaction between CL and histoincompatible targets.

The second conclusion issuing from our data was that allografted neonatal rats and cyclophosphamide-treated or neonatally thymectomized adult rats all

exhibited a reduced capacity for CMC. Calculations of CL in these kinds of rats, based on a one-hit theory (17, 34), disclosed that their lymphoid tissues contained a fraction of the number of CL enumerated in the lymphoid tissues of allografted unmanipulated adult rats. In the latter animals CL were calculated to be 0.5% in lymph nodes and 0.3% in spleens and peripheral blood. The calculated number tallies well with the calculated number of effector cells in lymphoid tissues of guinea pigs (37) and in peripheral blood of humans (38) with delayed hypersensitivity. Henney determined a similar small number per *H-2* antigen in sensitized mice (18), as did Brunner (39) and Cerottini et al. (16), employing a tumor graft model. Wilson et al. calculated 1–3% of reactive cells in a mixed lymphocyte culture system (36) by measuring uptake of thymidine-<sup>3</sup>H. This calculated number is two- to three-fold higher than the number of CL in a cytotoxic assay and is based on a different process, one of synthesis of nuclear DNA, which may not be equivalent to effector aggressor cells in an attack system.

The third conclusion derived from our data was that EAS and LABN-IgG delayed the development of CMC in rats with reduced immunologic capacity, e.g., in neonates and cyclophosphamide-treated or neonatally thymectomized adult rats. The period of deferral was about 4 days and appeared to be due to blockade of antigenic sites of the target graft. We have shown elsewhere that EAS and LABN-IgG were bound to allogeneic skin grafts and remained bound for 3–4 days (29). Those data and the ones reported here suggest “afferent blockade” in vivo. In vitro, “efferent blockade” apparently is operative simply because of the temporal design of the cytotoxic test. Blocking antibodies were bound to antigenic sites and thus prevented CL from attacking target cells. Preincubation of CL with EAS and LABN-IgG had no effect on release of <sup>51</sup>Cr from target cells. This result was not in accord with the reports of inhibition of CMC by blocking antibodies described by the Hellströms and their coworkers (40–42). By contrast, HARTD IgG containing xenogeneic antibodies to rat lymphocytes effectively inhibited CMC without complement.

In one series of experiments there was evidence that blocking antibodies bound to target antigens and normal (unsensitized) allogeneic lymphoid cells were slightly but significantly toxic to target cells. We cannot be certain that this is biologically important and, if it is, what mechanism might be involved. Perlmann and his colleagues have reported minor cytotoxic activity by normal human lymphocytes that have been activated by antigen-antibody complexes (6, 43). The complexes consisted of both xenogeneic chicken erythrocytes and rabbit antibodies. It is moot if their model can be related to our data with EAS and normal lymphocytes.

One can only speculate why different schedules of immunization elicited different levels of CMC. A single skin or renal graft was more effective than a single infusion of spleen cells or repeated skin and spleen cell grafts. Perhaps there was antigenic overload with the latter regimen, or perhaps BN spleen cells in Le recipients exerted a deleterious effect upon the development and expres-

sion of CMC. It was also possible that measurement of CMC 7 days after the last of several grafts was carried out on a day that was not optimal for CMC.

#### SUMMARY

Cell-mediated cytotoxicity (CMC) in spleens and lymph nodes of allografted rats was determined by release of  $^{51}\text{Cr}$  from labeled target cells incubated with aggressor lymphoid cells. CMC was first detected in grafted adult rats on day 5, peaked on days 7 and 8, and declined rapidly to background levels by days 9 to 11. In allografted neonates and in cyclophosphamide-treated or neonatally thymectomized adults CMC was a fraction of that observed in normal adult rats. Enhancing antibodies deferred *in vivo* peak activity of CMC in allografted neonates for 3-4 days, and blocked *in vitro* the action of aggressor lymphocytes by binding to target cells. Enhancing antibodies had no effect on the cytotoxicity of aggressor cells, but horse antibodies to rat thoracic duct cells inhibited *in vitro* CMC of aggressor cells.

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