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A First or Dominant Immunization. I. Suppression of Simultaneous Cytolytic T Cell Responses to Unrelated Alloantigens

By Donald A. Rowley and Renée M. Stach

From the Department of Pathology, The University of Chicago, Chicago, Illinois 60637

Summary

A first or dominant immunization with one antigen markedly inhibited specific cytolytic T lymphocyte (CTL) responses to a second unrelated alloantigen without suppressing antibody responses to other antigens. Suppression was induced rapidly, became systemic, and could be transferred passively with only serum. Suppression did not result from elimination of cells capable of responding to the second antigen. The mechanisms responsible for this "priority of the first response" may be the same that help protect the fetus during pregnancy, promote renal allograft survival after multiple blood transfusions, and prevent effective CTL-mediated immunity to variants of tumor cells or infectious agents that arise during tumor progression or chronic infections.

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Rejection of immunogenic tumors or elimination of various parasitic and viral infections depends ultimately on $CD8^+$ CTL directed against tumor or infected cells. Variants, however, may arise that express new or modified antigenic epitopes to which the host fails to respond. The molecular mechanism for generation of variants and escape from effective immunity has been described for various parasitic infections; in other diseases variants probably arise most frequently from mutations in malignant cells or in viruses, as may occur in an individual during chronic infection with HIV or in an infected population during epidemics of influenza (1–4). Interestingly, the host in which a variant arises may respond with high antibody titers to unrelated antigens (5), and the variant, though failing to induce immunity in the host of origin, is immunogenic in a normal individual (6–8). We report here development of a murine model for studying why variant tumor cells or infectious agents may fail to induce effective CTL-mediated immunity in an original host.

Materials and Methods

Mice and Cell Lines. C3H/HeN (MTV^-) females were purchased from the Frederick Cancer Research Production Facility (Frederick, MD). Mice were housed in a barrier facility and fed sterile water and food. Mice were 8–12 wk old when used experimentally. P815, EL4, and 1591-RE cell lines grown in MEM supplemented with 10% FCS were monitored regularly to be mycoplasma free. For convenience of presentation, C3H or 1591-RE tumor cells bearing $H2^k$ are referred to as A, C57BL or EL-4 tumor cells bearing $H2^b$ as B, and BALB/c or P815 cells bearing $H2^d$ as C.

Immunizations. Recipients under light ether anesthesia were injected in hind foot pads with 0.05 ml of cell suspension. Cells for immunization were dispersed washed allogeneic spleen cells, xenogeneic horse or sheep erythrocytes (HRBC or SRBC), or 1591-RE tumor cells harvested from tissue culture. Washed cells were suspended in sterile HBSS.

Assay for Cytotoxic Cells. Each popliteal lymph node and spleen was assayed separately in triplicate; in addition, for most experiments cells pooled from identically treated mice were also assayed in triplicate. CTL were measured using a 4-h ^{51}Cr release assay (9); each cell suspension was assayed at eight double dilutions beginning with an E/T cell ratio of 100:1; for ease of comparing differently treated groups we have recorded results for only three fourfold dilutions. Each number recorded is the percent ^{51}Cr release and is the mean for nine separate determinations; results for pooled cells are not recorded since at each dilution these results were within one dilution, plus or minus, of the number recorded. CTL in one way MLC were assayed similarly; each result is the mean for triplicate cultures assayed separately; the procedures for MLC and the preparation of nonadherent cells and dendritic cells (DC)[1] are described in the accompanying manuscript (10).

We have repeatedly confirmed that CTL present in lymph nodes or spleens of mice immunized as described above or recovered from MLC are $CD8^+$, i.e., treating the cells with αCD8 antibody and complement virtually eliminates cytolytic activity against appropriate target cells; also, the cells had very low cytotoxicity against YAC-1, a usual target for measuring NK activity (11). These results are not recorded since cytotoxicity reported for control target cells in the following experiments sets an appropriate value for comparing cytotoxicity against specific target cells.

Results

Preliminary Observations. The model developed for the present studies was derived from observations using an im-

[1] *Abbreviation used in this paper:* DC, dendritic cell.

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munogenic tumor designated 1591, which on transplantation into syngeneic mice grows initially but is subsequently rejected by the vast majority of mice; rejection requires $CD8^+$ lymphocytes directed against a dominant class I alloantigen of unknown origin (4). Very occasionally 1591 tumors grow progressively, but when this occurs tumors no longer express the dominant antigen, although other antigens are still expressed. For example, a variant of 1591 designated PRO4L grows progressively in normal mice challenged with 10^7 to 10^8 tumor cells, but is rejected by mice challenged with 10^6 tumor cells. Rejection of PRO4L is still dependent on $CD8^+$ lymphocytes, but by lymphocytes recognizing a different antigen (4).

Possibly a first response to 1591 suppressed effective immunity and permitted outgrowth of PRO4L in the original host. This notion was supported in an experiment showing that no tumors grew progressively in six of six mice injected with 10^7 1591 tumor cells or in six of six mice injected with 10^6 PRO4L tumor cells, but PRO4L tumors grew progressively in four of seven mice injected with a mixture of these numbers of tumor cells. Furthermore, mice injected several times with 1591 tumor cells alone failed to mount appreciable CTL responses to allogeneic spleen cells mixed with the tumor cells for subsequent injections (D. A. Rowley, unpublished results). These observations led to experiments that were designed to test the general possibility that a first or dominant immunization suppressed CTL responses to unrelated alloantigens. Because allogeneic cells were more readily available in large numbers and could be prepared in a more standardized way than tumor cells, they were used for the following experiments.

To approximate continuous antigenic stimulation of a tumor, allogeneic cells were injected every 2 or 3 d; in some experiments the numbers of cells were increased exponentially for the first two or three injections to mimic increasing antigenic challenge of growing tumors. Allogeneic cells were injected in a hind foot pad; CTL were measured in draining popliteal lymph nodes and spleens 1 or 2 d after the last injection of antigen. Elevated CTL responses occurred after three injections, i.e., 7–10 d after injections were begun. Levels of CTL activity fell rapidly $\geq$4 d after injections were stopped. CTL responses to three or four repeated injections were much higher than to the same dose of allogeneic cells given as a single dose 7 or 9 d earlier. CTL appeared at higher levels earlier in draining lymph nodes but responses were equivalent in lymph nodes and spleens after four or more injections of allogeneic cells. The mixing of two antigens did not diminish the CTL responses to either antigen and neither antigen stimulated significant responses to the other antigen (Fig. 1). The absence of crossreactions or competition persisted for at least eight injections given over 21 d, the longest interval tested. These results and those recorded in the following figures and table have been confirmed in two or more comparable experiments.

A First or Dominant Immunization Suppressed CTL to a Second Antigen. When mice that had received two or three injections of B cells were challenged with a mixture of B and C cells, CTL responses to C were very low; suppression to C was systemic since it did not matter whether the second antigen was mixed with the first antigen or was injected in the contralateral foot (Fig. 2). In other experiments, we found that the phenomenon was reproducible regardless of which strain of mouse was used as responders and which allogeneic cells were used as the first and second antigens. Also, suppression persisted for as long as injections of the first antigen were continued, 21 d being the longest time tested.

An interval between beginning the first and second immunizations was not necessary for establishing priority if one antigen was made dominant initially by dosage of antigen (Fig. 3, Exp. 1). Also, interaction between the cells used for immunization did not contribute to suppression, since a first immunization with B or C abolished responses to the other alloantigen when F_1 cells (cells from CB6 female mice, which were offspring of BALB/c male $\times$ C57 female) were used as the source of the second antigen (Fig. 3, Exp. 2).

Suppression of CTL but Not of Antibody Responses. A first immunization that so effectively suppressed CTL responses did not suppress antibody responses to xenogeneic erythrocytes used as a second antigen. For example, mice were injected three times with B spleen cells, as in the experiment in Fig. 2; three mice per group were then injected twice at 2-d intervals with a mixture containing 10^7 B and 10^8 SRBC, 10^7 B and 10^7 C cells, or 10^7 B, 10^7 C, and 10^8 SRBC per injection; controls included three additional groups not preinjected with B but receiving these injections. CTL responses to B and plaque-forming cells (PFC) to SRBC (12) were measured 4 and 6 d after the first injections of SRBC. All mice injected with SRBC had equivalent numbers of PFC: 6,000–10,000 direct (IgM) PFC and <1,000 indirect (IgG) PFC per node or spleen at day 4, and 20,000–60,000 indirect PFC per lymph node or spleen on day 6. All mice receiving the initial three injections of B had equivalent CTL responses to B.

In contrast, mice preinjected three times at 2-d intervals with xenogeneic erythrocytes failed to respond with CTL to allogeneic cells. For example, in one experiment mice were injected three times at 2-d intervals with a mixture containing 10^8 SRBC and 10^8 HRBC; the mice were then challenged three more times with a mixture containing $2\times$ 10^8 RBC and 10^7 C cells. 1 d after the last (7 d after the first) injection of C cells the mean CTL response of mice preinjected with RBC was 13, 7, and 5% lysis of C target cells at E/T ratios of 100:1, 25:1, and 6:1, respectively; the mean response of non-preinjected controls was 40, 20, and 11% lysis of C target cells at the same E/T ratios.

Cells from Suppressed Mice Respond Normally in Culture. Cells were obtained from spleens and lymph nodes of mice 1 or 2 d after the mice had received three or four injections of a first antigen; i.e., at a time when mice would not respond with CTL when challenged with a second antigen. The cells from such mice invariably responded to a second antigen in MLC as well as control cells from normal mice; also, cells from mice injected first with one antigen followed by injection of a mixture of the first and second antigen

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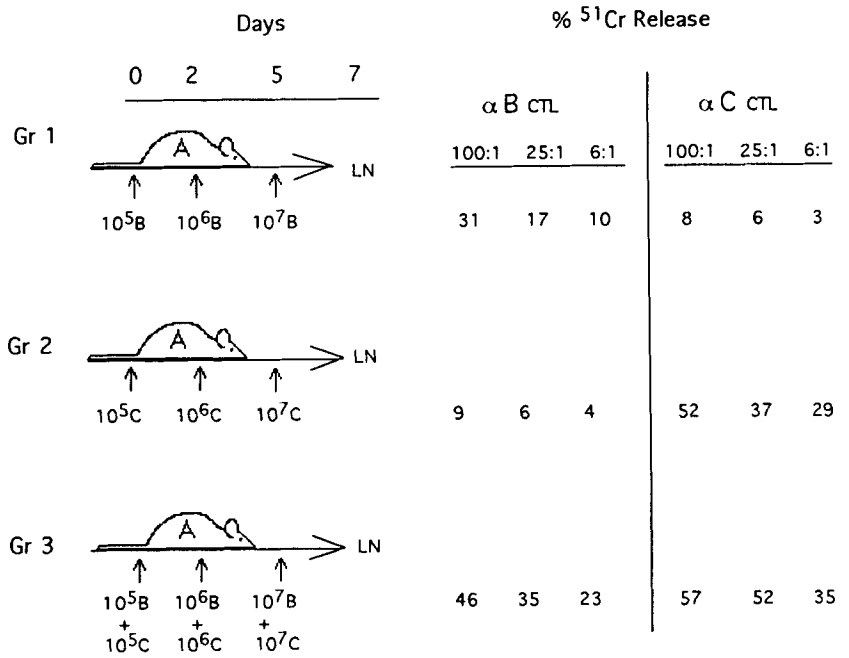

Figure 1. Absence of crossreaction or competition between alloantigens.

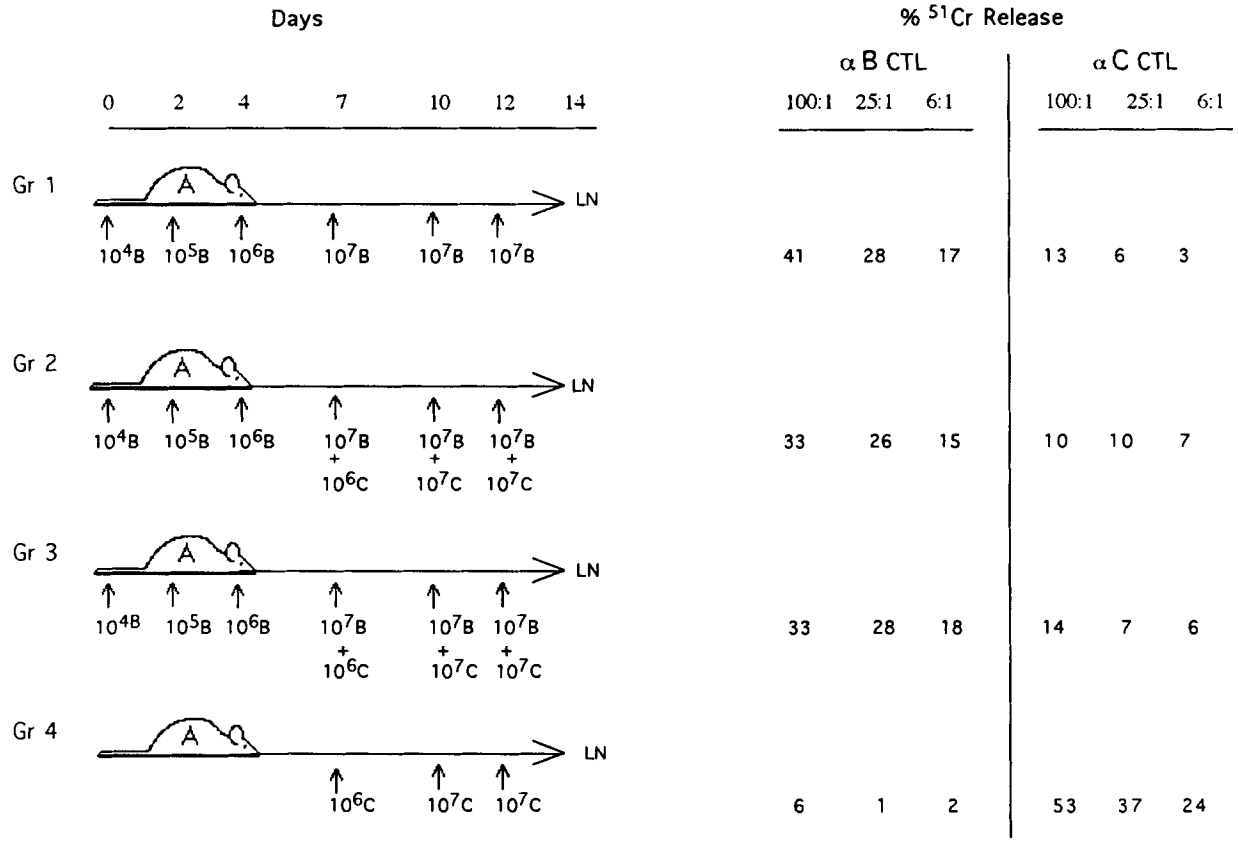

Figure 2. Prior immunization with allogeneic lymphoid cells prevents a CTL response locally and systemically to subsequent simultaneous immunization with unrelated allogeneic lymphoid cells. Antigen B was injected into right hindfoot pads. Antigen C was mixed with antigen B, group 2; antigen C was injected separately into left hind foot pads groups 3 and 4. Anti-B CTL are recorded for right popliteal lymph nodes, Groups 1, 2, and 3 and for left popliteal lymph nodes for group C. Anti-C CTL are recorded for right popliteal lymph nodes, groups 1 and 2, and for left popliteal and for groups 3 and 4.

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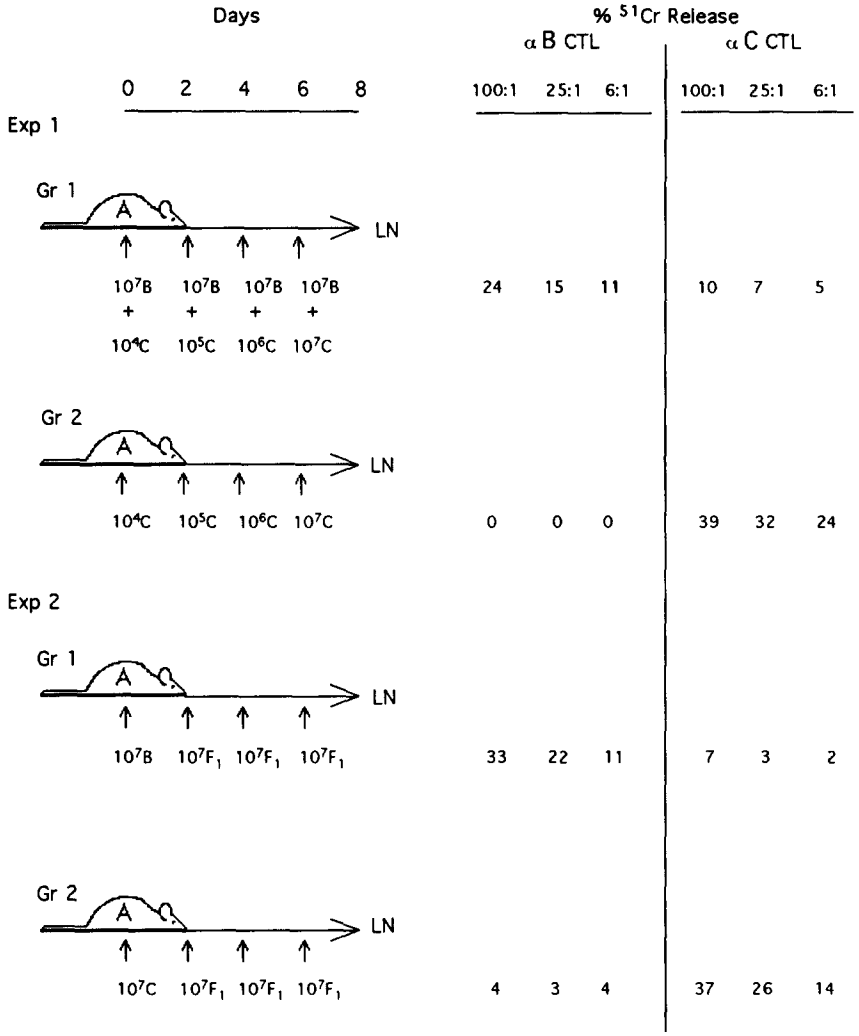

Figure 3. Dominant or first immunization with one antigen prevents CTL responses to a second alloantigen. In the first experiment, antigen B was made dominant by dosage of cells; antigens B and C were mixed, group 1. In experiment 2, B or C antigens were made dominant by first injection followed by injection of F_1 cells. For both experiments injections were in right foot pads and the responses recorded are for right popliteal lymph nodes.

responded normally to the second antigen in culture (e.g., Fig. 4, Exp. 1).

Conceivably, DC, which are required for CTL responses in cultures (11), might be altered by a first immunization; however, lymph nodes of mice injected with a first antigen or with a first antigen followed by a mixture of first and second antigen were as good a source of functional DC as normal spleens when tested using normal responder and irradiated stimulators cells, both depleted of DC (Fig. 4, Exp. 2).

Suppression by Passively Transferred Sera. In different experiments sera were obtained from A mice injected repeatedly with B or C cells. Normal A mice injected with the sera were challenged with three injections of either 10^7 B or 10^7 C cells. Surprisingly, serum raised against B not only suppressed CTL responses to B but also to C, and similarly, serum raised against C suppressed responses to both antigens. The amount of serum required to suppress CTL responses was quite large (0.5–1.0 ml whole serum per recipient); suppression was usually not as marked (approximately fourfold) as with active immunization, but suppression by unrelated immune sera was consistent in five consecutive experiments. These findings, illustrated in a single experiment (Table 1, Exp. 1), show that normal serum given passively to A mice sensitized to B had no effect on CTL responses to C, while both anti-B and anti-C sera suppressed responses to C. This lack of specificity of suppression was also observed using serum from mice injected repeatedly with xenogeneic erythrocytes (Table 1, Exp. 2).

Discussion

An extensive literature records that a first immunization with one antigen may suppress the antibody response to another antigen given subsequently (13–17). Selection of competing antigens, dosages of antigen, and timing of injections were usually critical for observing "antigenic competition," which was attributed in different models to: (*a*) consumption or exhaustion of critical cells or factors by the first immunization; (*b*) induction or activation of nonantigen-specific regulatory cells such as NK cells (18, 19); or (*c*) feedback inhibition by idiotypic or antiidiotypic antibody or effector cells directed against the suppressed epitopes or present on a

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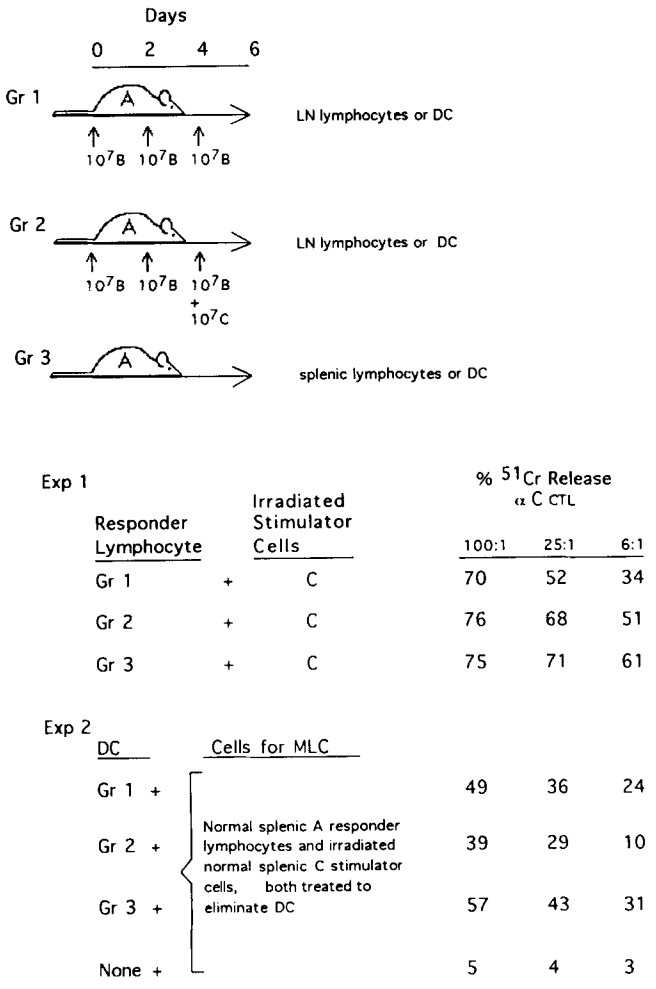

Figure 4. A first immunization with one alloantigen does not reduce or eliminate lymphocytes (Exp. 1) or dendritic cells (Exp. 2) required for primary CTL responses to a different alloantigen in vitro.

common carrier (20–26). In many of the models antibody responses were suppressed only moderately so that the biological significance of antigenic competition was often questionable. In contrast, the conditions for inducing suppression of $CD8^+$ CTL responses by a first or dominant immunization are not stringent and suppression is often marked without affecting antibody responses to a second antigen. Suppression is induced rapidly, becomes systemic early, can be passively transferred using serum from fully suppressed mice, and, as we report in the accompanying manuscript (10), is undoubtedly dependent on IgG. Suppression is inducible in two other strains of mice that have been tested and is independent of the combination of antigens used (data not reported), so that the phenomenon would appear to be general.

Table 1. *Passive Transfer of Suppression by Sera*

Exp.	Sera‡	Percent ^{51}Cr release of C target cells* 100:1	25:1	6:1
1	αB	18	5	2
	αC	6	1	1
	NMS	39	18	12
	None	39	16	12
2	αRBC	31	16	6
	NMS	66	38	16

* Normal A mice were immunized with 10^7 C cells injected every 2 d four times and killed on day 8. Mice receiving serum were given 0.6 ml whole serum on day 0 and 0.3 ml on day 3 (one half intravenously and one half intraperitoneally each time.) Results are the means for popliteal LN (three or four mice per group); each LN was assayed in triplicate against B and C target cells; the percent ^{51}Cr release for all samples against B target cells was <10% at E/T of 100:1.

‡ Donors were A mice that received four injections of 10^7 B or C cells (Exp. 1) or four injections of a mixture of 10^8 sheep and 10^8 horse erythrocytes (Exp. 2). Sera were given on the same day as donors were bled.

It seems remarkable that this mechanism of nonantigen-specific suppression of CTL responses should exist, but interestingly, a maternal antibody response to paternal HLA occurs early in pregnancy and an absence of this response may be associated with spontaneous abortion during the first trimester (27–29). Immunizing such women with paternal PBL may promote successful pregnancy (30–32). Thus, an early maternal antibody response to paternal antigens expressed by the fetus may help prevent maternal CTL responses directed against other antigens expressed later during fetal development. Operation of the same mechanism may explain why survival of renal allografts is enhanced in patients who have received multiple blood transfusions or were intentionally immunized with homologous erythrocytes (33, 34). On the other hand, the failure to develop CTL-mediated immunity to antigens expressed sequentially after a first or dominant immunization should be detrimental to individuals bearing immunogenic tumors or infected with organisms that give rise to variants expressing new or different antigenic epitopes. Thus, understanding how an early antibody response may suppress CTL responses to antigens subsequently expressed should help in developing new ways for promoting allograft survival and more effective immunity to variants.

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Address correspondence to Donald A. Rowley, Department of Pathology, The University of Chicago, 5841 South Maryland Avenue, MC1089, Chicago, IL 60637.

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