

ACTIVE SUPPRESSION AS A POSSIBLE MECHANISM OF TOLERANCE IN TETRAPARENTAL MICE

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The concept that immunological self-tolerance is solely due to the complete elimination of self-reactive clones has recently been challenged (1). Several alternative, although not mutually exclusive, explanations have been advanced. For example, Voisin (2), the Hellströms (3), and Ceppellini (4) and their associates have suggested that some types of tolerance may be effected by antibodies which somehow suppress antigenic recognition by immunologically competent cells. Also, Allison (5) and the Herzenbergs and their colleagues (6) have attributed other types of immunosuppressive activity to thymus-derived lymphocytes (theta-bearing T cells).¹ These alternative explanations have in common the notion that one immune cell population can interact with and suppress another, a concept which has received experimental support from studies using tetraparental mice. These mice, chimeric since the eight-cell stage because of whole embryo fusion, are operationally tolerant to themselves and both parental strains (7). Nonetheless they possess both immune cells capable of specifically destroying parental strain fibroblasts in vitro and specific serum blocking factors capable of preventing that destruction (8). These results have more recently been confirmed and extended using the mouse mixed lymphocyte culture technique (MLC) (9).

We now report that spleen cells from tetraparental mice do not respond to parental cells in vitro, and in addition are capable of preventing immunocompetent parental spleen cells from responding either to the opposite parent or to the tetraparental cells themselves in mixed lymphocyte culture. Furthermore, the ability of tetraparental mouse spleen cells to block in this fashion is immunologically specific and is not affected by treating them with antitheta antiserum.

Material and Methods

Tetraparental Mice.—These mice were derived from the fusion of C3H/HeJ and C57BL/10SnJ eight-cell stage embryos using techniques previously described (10-12). A detailed

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¹ *Abbreviations used in this paper:* B cells, non-thymus-derived cells; GVH, graft-vs.-host; MLC, mixed lymphocyte culture; PHA, phytohemagglutinin; T cells, thymus-derived lymphocytes.

protocol is available from T.G.W. on request. The C57BL/10(C57) and C3H/HeJ (C3H) parental strains differ at many loci, including the *H-2* histocompatibility locus, which is mainly responsible for MLC reactivity. All tetraparental mice used in this study were chimeric for coat color and hematopoietic phenotypes (12).

Mixed Lymphocyte Cultures (MLC's).—Cultures were performed by techniques previously described (13–15) with modifications for microtiter plate culture. In summary the medium consisted of RPMI-1640, 10% fresh heat-inactivated human serum, and 0.005 M *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethane sulfonic acid buffer. Spleen cells were used throughout this study. 2×10^6 total cells per well were incubated in Microtest II plates (Falcon Plastics, Oxnard, Calif.; no. 1030) for 60 h before a 4 h terminal incubation with 1 μ Ci [3 H]thymidine. Phytohemagglutinin (PHA)-stimulated cultures consisted of 1×10^6 cells. 1 μ l/ml of PHA-P (Difco Laboratories, Inc., Detroit, Mich.) was used with a 40 h incubation before terminal labeling and harvesting. A detailed protocol is available from S.M.P. on request.

One-Way Mixed Lymphocyte Cultures.—MLC's were made unidirectional by treating one of the two cell lines with mitomycin C, which allows those cells to serve as antigen while preventing them from proliferating (16). The cell line to be so treated was incubated for 40 min at 37°C in the standard medium supplemented with 2% normal human serum plus 25 μ g/ml mitomycin C (Nutritional Biochemicals Corp., Cleveland, Ohio) at a concentration of 10^7 cells/ml. The cells were then washed 3 times with a 10 min delay between washings. Control experiments showed these cells to be unresponsive in MLC.

Antitheta Antibody Treatment.—The AKR anti- θ C3H antibody was a gift of Dr. Carl Pierce. In order to confirm its specificity, the undiluted antiserum was incubated with 51 Cr-labeled C57 and C3H thymus, spleen, and bone marrow cells for 30 min at 4°C.² After the cells were washed, guinea pig complement at a final dilution of 1:4 vol/vol was added and the incubation was continued for 30 min at 37°C in the presence of DNase (2 μ m/ml). The three C57 cell populations released 85, 39, and 11% of the maximum releasable 51 Cr, respectively. The three C3H populations released 91, 37, and 7%, respectively. The cytotoxic activity was completely adsorbed by mouse brain tissue.

RESULTS

Previous experiments had indicated that although tetraparental spleen cells in culture had higher rates of spontaneous blastogenesis than either parental or F₁ hybrid cells, this rate was far less than that observed in MLC's between parental spleen cells (9). A possible interpretation of this result was the existence of a subpopulation among tetraparental spleen cells which is capable of preventing interaction between the two immunocompetent parental cell lines. To test this possibility, we mixed immunologically competent normal spleen cells of one parental strain type with tetraparental spleen cells. If the latter cells were capable of reducing their own expected endogenous reactivity they should similarly reduce the reaction of the added parental cells to the other parental cell line present in the tetraparental spleen cell suspension. Fig. 1 shows the results of 10 separate experiments in which C57, C3H, C57 \times C3H F₁ hybrid, C57 \leftrightarrow C3H tetraparental, and BALB/c spleen cells were mixed in various combinations. Results are expressed as a percentage of the response obtained using a simultaneously run parental MLC (C57 + C3H, 1:1), which is defined as 100% stimulation. The parental cells proliferated less in the pres-

² Pierce, C. 1972. Personal communication.

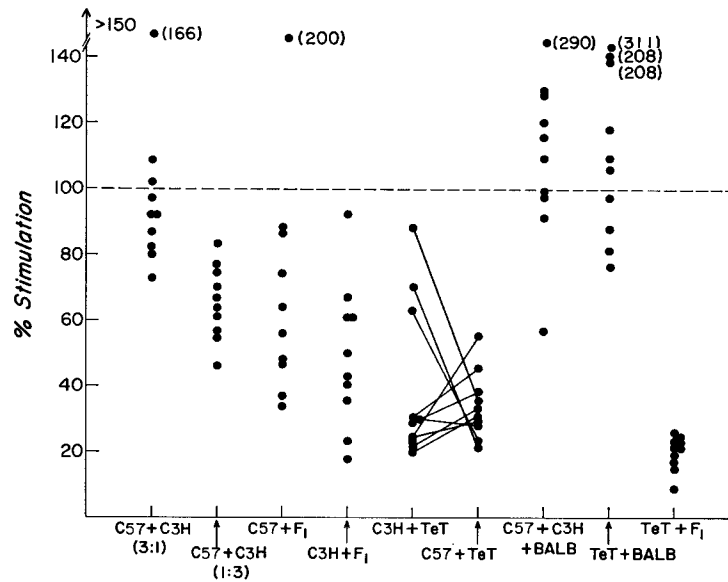


FIG. 1. The effect of tetraparental cells on the parental mixed lymphocyte culture response. This figure depicts data obtained when various combination of C57, C3H (C57 \times C3H) F₁ hybrid, C57 \leftrightarrow C3H, and BALB/c spleen cells are mixed in culture. The cell combinations are given along the abscissa. The ordinate represents the percent of stimulation (or incorporation of [³H]thymidine into the DNA) obtained compared with that obtained with C57 and C3H cells mixed in a 1:1 ratio (defined as 100% stimulation). Each point represents the normalized average of three cultures of the combinations (mixed in a 1:1 ratio, unless stated otherwise) shown on the abscissa. The lines drawn between tetraparental values connect the values obtained with a given tetraparental spleen when tested against the C3H (left point) and the C57 (right point) parental cell line. The other combinations represent various types of control combinations (see text).

ence of tetraparental spleen cells than they did when mixed in various proportions with the other parental cell line or with F₁ hybrid cells.

A few tetraparental spleen cell suspensions had only a weak ability to suppress a given parent, but the same population invariably suppressed strongly when mixed with the other parental cell line. This can be seen in Fig. 1, where in each case the point depicting the suppression of one parental cell type by a given tetraparental spleen cell preparation is connected by a line to the point showing how much suppression was obtained by another aliquot of the same tetraparental cell suspension when it was added to cells of the opposite parent.³

The reduction of proliferation by tetraparental spleen cells was specific for parental spleen cells. Spleen cells from an unrelated mouse (BALB/c) reacted

³ This could be a result of varying ratios of chimerism in a given tetraparental mouse, but there is no obvious relation between relative suppressive ability and the amount of hematopoietic chimerism as determined by hemoglobin mix.

as well with the C57 \leftrightarrow C3H tetraparental spleen cells as they did with mixtures of C57 and C3H cells. In these experiments, the tetraparental spleen cells were as capable of reacting with the BALB/c cells as were parental cells, judged by their reactivity in one-way MLC's (Table I). The tetraparental cells were not, however, capable of responding to either parental or F₁ hybrid cell lines. Third party cells were apparently responsive to tetraparental cells since the one-way (tetraparental plus mitomycin C-treated BALB/c) MLC response was usually less than the two-way (tetraparental plus BALB/c) reaction.

A second more stringent test of the in vitro suppressive ability of tetraparental spleen cells involved their effect upon an MLC established between normal parental strain spleen cells. This study was performed using a constant

TABLE I
Response of Tetraparental Cells in One-Way MLC

MLC combination		Stimulation index*, experiment number		
Cell 1	Cell 2	1	2	3
Tet‡	C57 _m §	1.8	2.0	1.6
Tet	C3H _m	1.4	1.0	1.2
Tet	(C57 \times C3H)F _{1m}	1.0	1.8	1.0
Tet	BALB/c _m	7.2	4.8	8.9
Tet	BALB/c	18.0	7.4	9.9
C57 + C3H(1:1)	BALB/c _m	7.8	5.9	9.8

* The stimulation index is defined as (³H]thymidine uptake in mixed culture)/(³H]thymidine uptake in unmixed control cultures).

‡ Tet, tetraparental mouse cells (C57 \leftrightarrow C3H).

§ m = mitomycin C treated. See Materials and Methods.

total number of cells consisting of varying percentages of either tetraparental (C57 \leftrightarrow C3H) or F₁ hybrid (C57 \times C3H) cells added to a mixture of normal parental cells (C57:C3H, 1:1). As depicted in Fig. 2, the substitution of small numbers of F₁ hybrid cells increased the total response. This result was similar to that seen with the addition of increasing numbers of mitomycin-treated target cells in one-way MLC's (14) and may be related to the increased amount of foreign H-2 antigen available for stimulation. It should also be pointed out that this increased response was observed despite a decreased number of cells theoretically capable of responding to antigen. Further increases in the percentage of F₁ cells eventually led to a decreased total response. The pattern obtained by varying the percentage of tetraparental cells in an identical manner was quite different. This cell population caused a decrease in total proliferation at all ratios tested, even those which caused stimulation when using the F₁ population. No difference between tetraparental and F₁ hybrid cells could be observed when they were added in the same manner to MLC's involving third

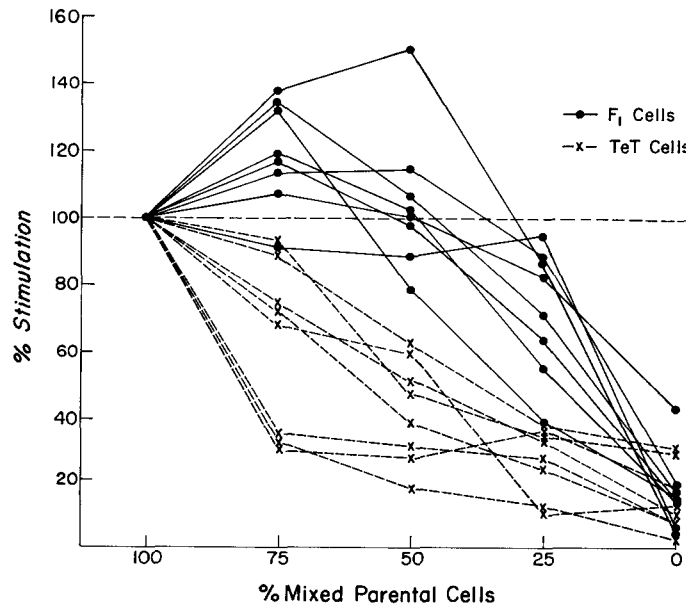


FIG. 2. The effect of various proportions of tetraparental and F_1 hybrid cells on the parental mixed lymphocyte culture response. Increasing proportions of F_1 hybrid (solid lines) or tetraparental (broken lines) spleen cells were added to a parental mixed lymphocyte culture (C57:C3H, 1:1), while the total cell number remained constant. The percentage stimulation, compared to control undisturbed parental MLC's (100% stimulation) is given on the ordinate. Control cultures were always run simultaneously with experimental cultures.

and fourth party interactions (Fig. 3). Also, harvesting the cultures at times earlier than day 3 ruled out the unlikely possibility that suppression (caused by *in vivo* sensitization [8]) on day 3 is really due to hyper-reactivity and early exhaustion of the cultures (Table II) (14, 15).

These observations are best explained by postulating that the tetraparental spleen cells can actively and specifically suppress parental mixed lymphocyte interaction.

Having demonstrated that tetraparental cells have suppressive ability *in vitro*, it now became possible to perform various manipulations on them to determine which cells are responsible for this activity and how they function. The next series of experiments was therefore designed to examine whether the cells possessing blocking ability are sensitive to anti- θ treatment *in vitro* before culture.

The anti- θ antiserum was tested for specificity as outlined in Materials and Methods above and for effectiveness in the following way. C57, C3H, and C57 \leftrightarrow C3H tetraparental spleen cells were treated with neat anti- θ antiserum and complement. The residual cell population was tested for its ability to respond to phytohemagglutinin (PHA) and allogeneic mitomycin C-treated cells.

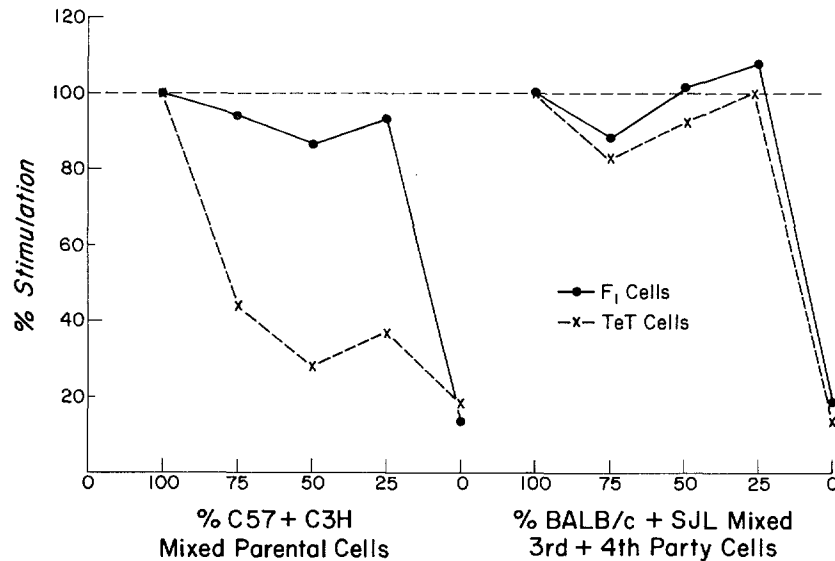


FIG. 3. The effect of various proportions of tetraparental and F_1 hybrid cells on parental and also on unrelated mixed lymphocyte cultures responses. Fig. 3 represents the same type of experiment as Fig. 2. On the left hand side, the differential suppression of a parental (C57:C3H, 1:1) MLC by tetraparental (C57 \leftrightarrow C3H) (broken line) compared with F_1 hybrid (C57 \times C3H) cells (solid line) is shown. On the right-hand side is shown the effect of cells from the same tetraparental and F_1 hybrid spleens on an MLC between unrelated spleen cell populations (BALB/c \times SJL). This experiment is representative of other similar experiments (not shown).

TABLE II
MLC Stimulation on Days 2 and 3

MLC combination		Stimulation index*	
Cell 1	Cell 2	Day 2	Day 3
C57	C3H	12.0	16.0
Tet*	C57	3.1	4.1
Tet	C3H	4.1	4.8
Tet	(C57 \times C3H) F_1	2.1	0.9
Tet	BALB/c	8.0	11.0
C57 + C3H(1:1)	BALB/c	5.3	7.8

* See footnotes to Table I.

The ability to respond mitotically to these two types of stimulation is thought to require the presence of thymus-derived lymphocytes (T cells) (17). The results are given in Table III; they indicate that anti- θ treatment of the responding cell population removed approximately 90% of the reactivity to PHA and all demonstrable reactivity to allogeneic cells.

In four separate experiments, tetraparental spleen cells were treated with

TABLE III
Effect of Anti- θ Antiserum and Complement on In Vitro Stimulation

MLC combination		Stimulation index* with anti- θ treatment	
Responding cell	Stimulant	Absorbed anti- θ ‡	Unabsorbed anti- θ
C57	PHA§	26.0	2.9
	BALB/c _m * [*]	7.3	1.0
C3H	PHA	32.0	3.7
	BALB/c _m	10.1	1.4
Tet*	PHA	41.0	3.1
	BALB/c _m	8.2	1.5

* See footnotes to Table I.

‡ Anti- θ absorbed with mouse brain, 20% vol/vol.

§ Phytohemagglutinin.

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TABLE IV
Effect of Anti- θ Treatment on MLC Suppression by Tetraparental Cells

MLC Combination			% Stimulation of suppressed parental MLC*							
Cell 1	Cell 2	Exp. no.:	Absorbed anti- θ ‡				Unabsorbed anti- θ			
			1	2	3	4	1	2	3	4
Tet§	C57		18	38	23	36	26	28	18	24
Tet	C3H		26	22	63	88	33	16	47	14

* Compared with un-suppressed parental MLC (C57:C3H, 1:1), which is taken as 100% stimulation.

‡ Absorbed with mouse brain.

§ See footnotes to Table I.

anti- θ antiserum in an identical manner. As a control, separate aliquots of tetraparental spleen cells were treated with the anti- θ antiserum which had been previously adsorbed (3 times) with a 20% vol:vol preparation of fresh mouse brain tissue. When these two cell populations were individually mixed with parental or F₁ hybrid cells, both suppressed equally and as well as untreated tetraparental cells (Table IV). These results indicate that, within the limits of the experiments, the tetraparental suppressor cells are not sensitive to the action of anti- θ antibody.

DISCUSSION

A number of recent experiments indicate that some immune cell-to-cell interactions, rather than being synergistic, are in fact antagonistic. The data presented here indicate that mice chimeric since the eight-cell stage with two histoincompatible cell lines contain cells in the spleen which are capable of specifically suppressing immunocompetent adult parental cells in mixed lymphocyte culture. This finding is similar to observations made by Ceppellini in

studies of human fetal-maternal cell interaction in vitro (4).⁴ He found that maternal cells could suppress the proliferation of paternal lymphocytes in response to newborn cells in MLC. The current work also confirms previous work on tolerance in tetraparental mice (8, 9) and provides a possible means of characterizing the suppressor cell population because the suppression can be detected in vitro. As a first step in this direction we have shown that treatment of tetraparental spleen cells with an otherwise effective antitheta antiserum and complement has no effect on the suppressive activity of these cells. The simplest interpretation of this result is that the suppression is carried out by cells which are thymus independent. Further support for this notion derives from the observation that blocking factor found in the serum of tetraparental mice behaves like an immunoglobulin (9), which would argue for its being produced by non-thymus-derived (B) cells. However, these experiments have not ruled out other possibilities. One is that the suppressor cells are antitheta-resistant T cells. Another is that the blocking substance is made by T cells and stored in other cells. Further use of the methods described here should allow one to distinguish between these and other possibilities.

The current experiments, and others as well (2, 8, 9, 18), make it reasonable to postulate active and continuous suppression as a means of tolerance in mouse chimeras composed of two histoincompatible cells lines. It should be noted, however, that experiments done with rat chimeras in a variety of laboratories do not provide evidence for suppression as a mechanism of tolerance. For example, Wilson and Nowell reported that tolerant cells do not seem to interfere with nontolerant cells in MLC (19). Atkins and Ford reported that chimeric cells and/or chimeric serum had little, if any, suppressive effect on immunocompetent cells mediating graft-vs.-host (GVH) reactions (20). Recent experiments of Elkins may help to elucidate the situation in rat chimeras (21). He found, as did Atkins and Ford, that chimeric cells from rats would neither cause nor suppress a GVH reaction. However, if the chimera was challenged beforehand with nonchimeric host strain cells, then the "chimeric" cells readily suppressed GVH reactions. This suggests that the suppressor cells can be increased in effectiveness by challenge. Perhaps they are usually below detectability in rat chimeras, but are more easily detectable in the systems described here and elsewhere. We have also observed that the level of blocking factor in the serum of tetraparental mice varies considerably depending on a number of as yet poorly understood variables (unpublished observations).

The foregoing discussion makes apparent the need to clarify how tolerance to naturally occurring self-antigens obtains in nonchimeric individuals. Burnet has recently observed that his forbidden clone hypothesis is not yet proven to be the correct explanation for natural tolerance (22). We have proposed that it will be true for the set of self-antigens present on reactive lymphocytes in

⁴ Ceppellini, R. 1972. Personal communication.

nonchimeric individuals, based on the differences between tetraparental and F₁ hybrid mice (9). Micklem (23), Cohen et al. (24, 25), and Pierce (26) have provided examples in which "forbidden clones" may exist in normal animals. It remains to determine whether the forbidden clone hypothesis, or active suppression, or some combination of the two, is the true explanation for self-nonreactivity.

SUMMARY

Previous work has indicated that tetraparental mice, chimeric since the eight-cell stage because of embryo fusion using histoincompatible strain combinations, possess autospecific immune cells and blocking antibodies. Although this phenomenon has been demonstrated *in vitro*, it may have relevance to the self-tolerance shown by these mice *in vivo*. The experiments described here indicate that spleen cells from tetraparental mice can block mixed lymphocyte reactions between the two parental cell types, but not between unrelated strains. Furthermore, this suppressive ability is not affected by an otherwise effective treatment of the tetraparental spleen cells with anti- θ antibody and complement. The *in vitro* experimental system elaborated here should help to characterize the cell type responsible for the suppression.

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