# INDUCTION OF SPECIFIC IMMUNE UNRESPONSIVENESS WITH PURIFIED MIXED LEUKOCYTE CULTURE-ACTIVATED T LYMPHOBLASTS AS AUTOIMMUNOGEN

## III. Proof for the Existence of Autoanti-Idiotypic Killer T Cells and Transfer of Suppression to Normal Syngeneic Recipients by T or B Lymphocytes*

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We have recently been able to induce specific immunological unresponsiveness towards major histocompatibility antigens in adult, immunocompetent animals by using as immunogen, autologous, purified T lymphoblasts obtained via in vitro activation in mixed leukocyte cultures (1–3). Repeated injections of T blasts in adjuvant will lead to selective inability of many blast-immunized animals to react against the relevant alloantigens in mixed leukocyte culture (MLC)[1], cell-mediated lympholysis (CML), or graft-versus-host assays (1–3). Immunological tolerance as evidenced by prolonged survival of tissue grafts can be demonstrated in the autoblast-immunized animals. H. Binz, L. C. Andersson, and H. Wigzell, unpublished observations. The suppression is specific and once induced would seem to be quite long lasting. Preliminary results suggest that the same autoblast principle may be applied in induction of immune unresponsiveness against conventional antigens. (H. Binz and H. Wigzell, unpublished observations).

The detailed mechanisms underlying this induction of specific-immune tolerance in immunocompetent individuals will be analyzed in the present article. The initial reasoning behind the above blast protocol was based on our knowledge that immunocompetent T cells have idiotypic receptors on their surface signifying their specific immunocompetence (4). Anti-idiotypic immunity raised in a conventional manner could be shown to selectively eliminate the idiotype-positive cells and in parallel leading to elimination of specific-immune reactivity against the relevant alloantigens. Later, we found it possible to induce autoanti-idiotypic antibodies against the T-cell receptors with specificity for alloantigens by using as immunogen, pure, soluble receptors introduced in a polymerized form (5). To make the approach more applicable to a system where anti-idiotypic immunosorbants would be hard to come by (signifying such immunosorbants were necessary in these earlier experiments to obtain the purified receptors), we

* Supported by the Swiss National Science Foundation, grant no. 3.688-0.76, the Swedish Cancer Society and National Institutes of Health, grant AI.CA. 13485-01.

[1] *Abbreviations used in this paper:* D-PBS, Dulbecco's modified phosphate-buffered saline; EHAA, Eagle's high amino acid medium; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; [$^3$H]TdR, tritiated thymidine; MLC, mixed leukocyte culture; CML, cell-mediated lympholysis.

then tried to induce the same kind of autoanti-idiotypic immunity but now with the antigen-specific, idiotype-positive T cells as immunogens (1–3).

We know that several autoblast-immunized animals will indeed produce autoanti-idiotypic antibodies of expected specificity (1–3). However, it is not clear whether this autoanti-idiotypic immunity is the dominating factor in inducing the specific unresponsiveness and, if so, whether this is expressed on T cells as well as on B cells. Furthermore, the autoblast immunization procedure carries a second specific element besides the idiotypic receptors, namely soluble alloantigens derived from stimulator cells and being present on some MLC T blasts (6). It could thus be argued that such alloantigenic material when transferred with the T blasts may serve as a potent inducer of alloantigen-specific suppressor T cells. These hypothetical antigen-specific suppressor cells may then, rather than the autoanti-idiotypic immune response, be responsible for the observed-specific unresponsiveness. In the present article we would like to present data proving autoanti-idiotypic immunity at the T-cell level in the blast-immunized animals. Furthermore, the specific unresponsiveness could be shown to be transferred by both T and B lymphocytes to normal, syngeneic animals. Finally, when analyzed for specificity it could be shown that these suppressor cells had specific affinity fractionation to relevant, idiotype-positive antibody molecules. The practical and theoretical consequences of the present results will be discussed.

## Materials and Methods

*Mice.* Mice of the inbred strains C57BL/6J, CBA/J, and DBA/2J were purchased from The Jackson Laboratory, Bar Harbor, Maine, or bred in our own colony. Adult mice of uniform sex were used within the experiments.

*Lymphocyte Preparations.* Spleens were aseptically removed and single cell suspensions were prepared with a stainless steel mesh by using Dulbecco's modified phosphate-buffered saline (D-PBS) as a medium. Cells were washed once and erythrocytes lysed by hypotonic shock by using 9 parts of distilled water and 1 part of ×10 D-PBS. Cells were washed again and resuspended in D-PBS or culture medium.

*MLC.* MLCs were performed in flat bottom microtiter plates (Cook M220-29ART, Greiner, Nürtingen, Germany), by using $0.25 \times 10^6$ responder lymphocytes and $0.5 \times 10^6$ 2,000 rads irradiated stimulator cells. Eagle's high amino acid (EHAA) medium (7) complemented with 0.5% fresh normal mouse serum was used as a culture medium.

Cultures were pulsed for 6 h with 1 μCi of tritiated thymidine ([$^3$H]TdR) (The Radiochemical Center, Amersham, Great Britain, sp act 40–60 Ci/mmol).

Cultures were harvested on a Skatron collector (Skatron, Lierbyen, Norway) on glassfiber filters and counted in 2 ml of scintillation fluid in plastic scintillation vials.

For the preparation of large quantities of specific T lymphoblasts or killer cells, MLC was performed in Falcon 3013 flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) with the same medium as above. 15 ml of a cell suspension containing $1.25 \times 10^6$ responder cells and $2.5 \times 10^6$ 2,000 rads irradiated stimulator cells per ml were added to each flask. Cultures were harvested on day 4 or 5 after initiation of the MLC.

*CML.* CML was performed in V-bottom microtiter plates in 200 μl of EHAA medium supplemented with 5% heat-inactivated fetal calf serum (FCS). Assays were carried out in triplicate. Each well contained $1 \times 10^5$ or $1 \times 10^6$ effector cells and $1 \times 10^4$ $^{51}$Cr-labeled target cells. Plates were incubated for 4 h at 37°C in 5% $CO_2$ in air. Maximum release figures were determined by 100 μl of 0.4% NP-40 detergent (Nonidet-P40, Fluka, Buchs, Switzerland). Percent cytotoxicity (% CML) is expressed in the following form

$$100 \times \frac{\text{Experimental} - \text{spontaneous } ^{51}\text{Cr-release}}{\text{Maximum} - \text{spontaneous } ^{51}\text{Cr-release}}.$$

The following target cells were used: for H-2$^b$, EL-4; for H-2$^d$, P-815; and for H-2$^k$, Con A blasts harvested at day 3 of culture.

*Purification of T Lymphoblasts from MLC with 1 g Velocity Sedimentation.* MLC cells were pooled in 50-ml Falcon tubes and centrifuged for 10 min at 400 *g*. Cells were washed again in D-PBS and resuspended in D-PBS containing 5% FCS. Cells were then applied on a linear 15–30% FCS gradient by using D-PBS as diluent (8). The gradient was harvested after 4–5 h at 4°C in 15-ml tubes and the blast fractions (determined under the microscope) pooled and washed four times in 50-ml Falcon tubes.

*Immunization of C57BL/6 Mice with C57BL/6 Anti-CBA MLC T Lymphoblasts.* Each C57BL/6 mouse received 1 × 10$^7$ purified C57BL/6 anti-CBA MLC T lymphoblasts in 0.2 ml of D-PBS and Freund's adjuvant intraperitoneally. Complete Freund's adjuvant was used for the first injection and incomplete adjuvant was used for the boosters which were given in 3-wk intervals. Animals were sacrificed 10 days after the last injection. As a control, C57BL/6 mice were injected with Freund's adjuvant alone.

*Treatment of Lymphocytes with Different Antisera and Complement*

ANTI-THY-1.2 AND C'. AKR anti-$C_3H$ (anti-Thy-1.2) antiserum was purchased from Searle (High Wycombe, Great Britain) and used at a concentration of 1:5. 1 ml of diluted antiserum was incubated with 5 × 10$^7$ spleen cells. Incubation was carried out at room temperature for 30 min. The serum was removed by centrifugation and the cells resuspended in 1 ml of 1:5 diluted rabbit complement and incubated for 30 min at 37°C with occasional shaking. Cells were washed three times thereafter. Lysis was according to expected T-cell percentages. The remaining viable cells were screened for Ig-negative cells by incubating the cells with fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse Ig antiserum. The preparations showed less than 2% of contamination with Ig-negative lymphocytes.

TREATMENT OF CELLS WITH ANTI-Ly ANTISERA. Anti-Ly-1.2, 2.2, and 3.2 were kindly provided by Dr. E. Boyse and Dr. H. Cantor. Anti-Ly-1.2 was used at a final concentration of 1:40. Anti-Ly-2.2 and 3.2 were used together and at a final concentration of 1:30. 5 × 10$^7$ cells were incubated with 1 ml of diluted antiserum for 30 min at room temperature, spun down, and the pellet was resuspended in 1:5 diluted rabbit complement. Incubation with complement was performed for 30 min at 37°C with occasional shaking. D-PBS was used as a diluent.

TREATMENT OF CELLS WITH NORMAL MOUSE SERUM. This was done as described for the treatment of the cells with anti-Thy-1.2 antiserum.

*Preparation of Alloantisera.* Alloantisera of specificity C57BL/6 anti-CBA and C57BL/6 anti-DBA/2 were elicited in C57BL/6 mice initially primed with CBA or DBA/2 skin grafts. Mice were boosted 3 wk after rejection of the skin graft by injecting 5 × 10$^7$ CBA or DBA/2 spleen cells followed by the same dose 1 mo later and were bled 10 days thereafter.

*Purification of T Lymphocytes.* Spleen cells were passed over Degalan-Ig anti-Ig columns (Degalan V-26, Degussa, Frankfurt, Germany) as described previously (9). These T-cell preparations showed less than 2% of contamination of Ig-positive lymphocytes as judged by FITC-labeled rabbit anti-mouse antiserum.

*Experimental Plan.* Fig. 1 summarizes the experimental plan. A large group of C57BL/6 mice immunized four times with 10$^7$ C57BL/6 anti-CBA/J purified T lymphoblasts at 3-wk intervals followed by killing the mice 10 days after the last immunization served as cell donors representative for autoblast immunized individuals. Spleen cells were pooled and were then used according to the following scheme.

EXP. 1. Samples were tested for reactivity against CBA/J and DBA/2 stimulator cells in MLC. This would demonstrate the degree of specific unresponsiveness against CBA/J stimulator cells as caused by the autoblast immunization procedure.

EXP. 2. T cells were prepared from the spleen cell pool and tested directly for CML activity against various targets as denoted 2a–f. This part of exp. 2 was designed to screen for cytolytic T cells with specificity either for conventional alloantigenic targets or for autologous T blasts carrying the relevant idiotypic receptors as targets (the positive control being 2d, whereas 2e and 2f would serve as specificity control in case of significant cytolysis). To further test for specificity of lytic T cells, exp. 2g–i were included where specific or unrelated cold T target blasts or idiotypic alloantibodies were added as attempted specific inhibitors of cytolysis. Exp. 2 would thus test for presence of anti-idiotypic, cytolytic T cells in autoblast-immunized animals.

EXP. 3–9. This series of experiments was designed to test whether an induced unresponsive-

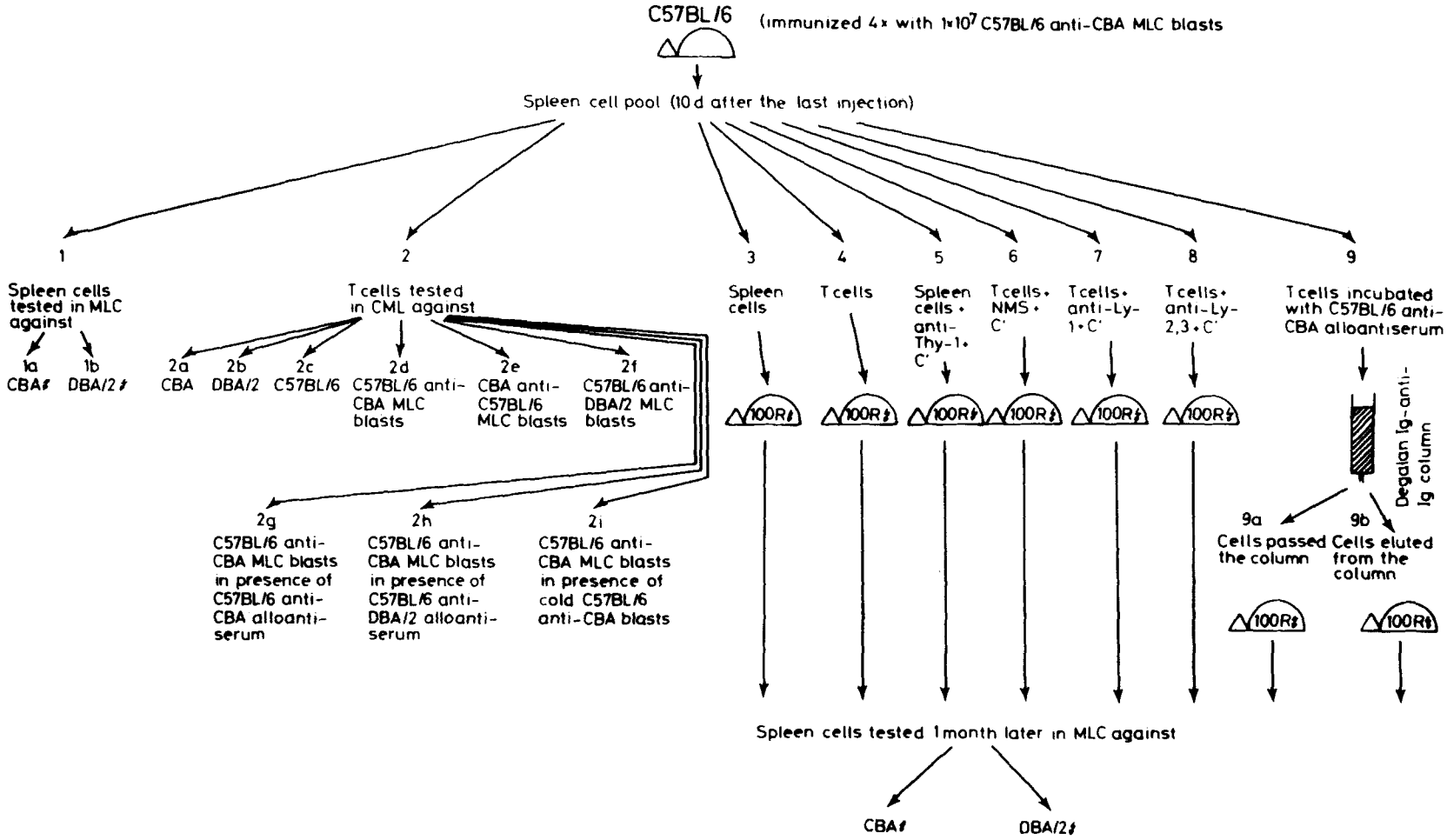
C57BL/6
(immunized 4× with 1×10⁷ C57BL/6 anti-CBA MLC blasts
Spleen cell pool (10 d after the last injection)
1
Spleen cells tested in MLC against
1a CBA♯
1b DBA/2♯
2
T cells tested in CML against
2a CBA
2b DBA/2
2c C57BL/6
2d C57BL/6 anti-CBA MLC blasts
2e CBA anti-C57BL/6 MLC blasts
2f C57BL/6 anti-DBA/2 MLC blasts
2g C57BL/6 anti-CBA MLC blasts in presence of C57BL/6 anti-CBA alloantiserum
2h C57BL/6 anti-CBA MLC blasts in presence of C57BL/6 anti-DBA/2 alloantiserum
2i C57BL/6 anti-CBA MLC blasts in presence of cold C57BL/6 anti-CBA blasts
3
Spleen cells
4
T cells
5
Spleen cells + anti-Thy-1 + C'
6
T cells + NMS + C'
7
T cells + anti-Ly-1 + C'
8
T cells + anti-Ly-2,3 + C'
9
T cells incubated with C57BL/6 anti-CBA alloantiserum
Degalan Ig-anti-Ig column
9a Cells passed the column
9b Cells eluted from the column
100R♯
Spleen cells tested 1 month later in MLC against
CBA♯
DBA/2♯

FIG. 1.

ness in autoblast-immunized C57BL/6 mice against CBA/J alloantigens could be transferred into normal C57BL/6 recipients. These recipients were irradiated with 100 rads before inoculation with presumed suppressor cells to ensure a more efficient take of the transferred cells but without causing any drastic damage to the normal lymphocytes of the recipient (10, 11). The various designs included an analysis as to whether T and/or B lymphocytes alone could transfer suppression and did also include an attempted analysis as to the Ly phenotype of suppressive T lymphocytes. In exp. 9, a crucial experiment was included by applying principles that were previously fruitful when attempting to fractionate T lymphocytes according to their idiotypic receptors, allowing recovery of T cells in a functionally intact form (12). Here, T cells from the blast-immunized animals were incubated with idiotypic C57BL/6-anti-CBA alloantiserum. If such suppressor T cells were indeed specific for the idiotypic alloantibody molecules, they should bind such immunoglobulin molecules. After washing, these antiserum-incubated cells would then be filtered through a second anti-Ig column (the T cells were initially filtered through an anti-Ig bead column) and anti-idiotypic T cells would now be retained in the column due to the coating with idiotype-positive Ig molecules. Passed and mechanically eluted T cells could then be analyzed for ability to transfer suppression into normal recipients. This latter test could thus directly demonstrate the actual specificity of the suppressor T cells in the present system to be autoanti-idiotypic, providing a positive outcome of the transfer tests.

## Results

*Specific Unresponsiveness of Spleen Cells from Autoimmunized C57BL/6 Mice to CBA Alloantigens as Measured in MLC (1a and 1b in Experimental Plan).* 45 C57BL/6 mice immunized four times with $1 \times 10^7$ purified C57BL/6 anti-CBA MLC T lymphocytes emulsified in Freund's adjuvant in 3-wk intervals were sacrificed 10 days after the last injection and their spleen cells pooled. This pool was first tested for its reactivity in MLC against CBA and DBA/2 stimulator cells. DBA/2 served as a third party control.

Table I shows that the proliferative response of spleen cells from autoimmunized C57BL/6 mice against CBA stimulator cells was drastically reduced when compared with the control involving spleen cells from normal C57BL/6 mice. The reactivity against DBA/2 stimulator cells on the other hand was hardly touched. This experiment showed us that the autoimmunization was successful in a similar manner, reported previously (1–3).

*Specific Unresponsiveness of T Lymphocytes from Autoimmunized C57BL/6 Mice to CBA Alloantigens as Measured in CML (2a–2c in Experimental Plan).* In a second step, we also tested the cytolytic capacity of T lymphocytes from autoimmunized C57BL/6 mice after stimulation for 6 days in MLC with either CBA, DBA/2, or C57BL/6 stimulator cells. As a control, T lymphocytes from normal C57BL/6 mice were stimulated against the same alloantigens. Table II summarizes this experiment.

As shown, specific reduction in cytolytic activity against CBA targets does exist among the T-cell pool. Again, this supports and extends earlier observations in the autoblast system (1–3).

*Spleens for Autoimmunized C57BL/6 Mice Contain Autoanti-Idiotype-Specific Killer T Cells (2d–2g and 2i in Experimental Plan).* In previous experiments we found anti-idiotypic antibodies in some sera of mice which have been immunized with syngeneic MLC T lymphoblasts (1–3). However, specific suppression of the response against the corresponding alloantigens as measured in MLC could be demonstrated in close to all the autoimmunized animals. Our test for the presence or absence of anti-idiotypic antibodies was maybe not sensitive enough (protein A assay [9]). Alternatively, there may also be T-cell-

TABLE I

*Specific Unresponsiveness of Autoimmunized C57BL/6 Mice to CBA Alloantigens as Measured in MLC*

| Responder cells derived from | [$^3$H]TdR incorporation of mixture with CBA stimulators. Mean cpm of triplicates ± SE* | | [$^3$H]TdR incorporation of mixture with DBA/2 stimulators. Mean cpm of triplicates ± SE* | | [$^3$H]TdR incorporation of responder cells alone. Mean cpm of triplicates ± SE* | |
|---|---|---|---|---|---|---|
| | Day 4 | Day 5 | Day 4 | Day 5 | Day 4 | Day 5 |
| Normal C57BL/6 | 192,479 ± 8,458 | 180,717 ± 7,571 | 237,138 ± 4,820 | 191,342 ± 6,830 | 2,871 ± 98 | 2,203 ± 137 |
| C57BL/6 four times immunized with C57BL/6 anti-CBA MLC T blasts | 18,264 ± 2,322 | 17,942 ± 970 | 193,238 ± 2,354 | 156,837 ± 5,563 | 3,513 ± 178 | 3,349 ± 199 |

* MLC was performed in flat bottom microtiter plates as described under Material and Methods. Cultures were pulsed for 6 h with 1 $\mu$Ci of [$^3$H]TdR. Autoimmunized C57BL/6 mice were injected four times with $1 \times 10^7$ of C57BL/6 anti-CBA purified MLC T lymphoblasts in 3-wk intervals. Animals were killed 10 days after the last injection.

TABLE II

*Lack of Generation of Cytotoxic T Lymphocytes in Autoimmunized Mice*

| Cytotoxic T lymphocytes generated in MLC from | CML of H-2$^b$ target cells* | CML of H-2$^d$ target cells* | CML of H-2$^k$ target cells* |
|---|---|---|---|
| | % | % | % |
| Normal C57BL/6 mice | −0.84 | 56.4 | 49.9 |
| C57BL/6 mice four times immunized with C57BL/6 anti-CBA MLC T blasts | −0.32 | 55.2 | 1.9 |

* Cytotoxic T lymphocytes were elicited in MLC in 3013 Falcon flasks. Cells were harvested on day 6 and CML was performed as described under Material and Methods. Parameters otherwise as described for Table I. El-4 tumor cells served as target cells for H-2$^b$. P-815 for H-2$^d$ and Con A-induced CBA blasts for H-2$^k$. Killer to target cell ratio 10:1, mean of triplicates.

mediated mechanisms as suppressor factors in some autoimmunized mice. We have previously been able to demonstrate anti-idiotypic killer spleen lymphocytes in autoblast-immunized mice (1). We have here further investigated this killer system and could demonstrate that there indeed exists cytolytic T lymphocytes with specificity for idiotypic determinants in specifically activated T target lymphoblasts in autoblast immune spleens.

T lymphocytes from the spleen cell pool of C57BL/6 mice immunized with C57BL/6 anti-CBA MLC T lymphoblasts were prepared as described in Material and Methods. These T cells were directly incubated with $^{51}$Cr-labeled C57BL/6 anti-CBA, C57BL/6 anti-DBA/2, or CBA anti-C57BL/6 purified MLC T lymphoblasts (2d–2f in experimental plan). As a control, T lymphocytes from C57BL/6 mice injected only with Freund's adjuvant were used. As shown in Table III, T lymphocytes from autoimmunized C57BL/6 mice contained T cells able to lyse C57BL/6 anti-CBA MLC T lymphoblasts. This killing was specific because C57BL/6 anti-DBA/2 or CBA anti-C57BL/6 MLC T lymphoblasts were not lysed. To further prove the specificity of this killing, T lymphocytes from autoimmunized C57BL/6 mice were incubated with $^{51}$Cr-labeled C57BL/6 anti-CBA MLC T lymphoblasts in the presence of cold blasts of the very same

TABLE III
*Demonstration of Idiotype-Specific Killer T Lymphocytes*

| Cytolytic T lymphocytes derived from | CML performed in the presence of | CML of C57BL/6 anti-CBA blasts* | CML of C57BL/6 anti-DBA/2 blasts* | CML of CBA anti-C57BL/6 blasts* |
|---|---|---|---|---|
| | | % | % | % |
| Normal C57BL/6‡ | – | −0.11 | 0.55 | −0.39 |
| C57BL/6 four times immunized with C57BL/6 anti-CBA blasts | – | 72.52 | 1.47 | −0.17 |
| " | Normal C57BL/6 serum | 63.54 | | |
| " | C57BL/6 anti-CBA serum | 9.22 | | |
| " | C57BL/6 anti-DBA/2 serum | 59.75 | | |
| " | C57BL/6 anti-CBA MLC blasts not labeled | 18.73 | | |
| " | C57BL/6 anti-DBA/2 MLC blasts not labeled | 60.08 | | |

* CML was performed as described under Material and Methods. Killer to target cell ratio 100:1. In some of the experiments normal mouse serum or alloantisera of specificity as indicated was used instead of EHAA medium. In the experiments with cold target cells, labeled and cold targets were suspended in 100 μl medium, 10,000 of each. Mean of triplicates.

‡ Injected four times with Freund's adjuvant only.

specificity. As shown in Table III, the killing could now be inhibited. These experiments show that the spleen of autoimmunized mice contains cytolytic T lymphocytes which can recognize idiotypic determinants on specifically activated T lymphoblasts known to be present in high density on these cells. H. Binz and H. Wigzell, unpublished observations.

T lymphocytes with capacity to react against a given set of alloantigens and alloantibodies produced in the same strain and directed against the same antigenic determinants express in part the same or very similar idiotypic determinants (9). We therefore added the question whether a C57BL/6 anti-CBA alloantiserum could also specially inhibit the T cells from autoimmunized C57BL/6 mice with lytic specificity for C57BL/6 anti-CBA MLC T lymphoblasts as described above. C57BL/6 anti-DBA/2 alloantiserum was used as control. As shown in Table III (2g and 2 h in experimental plan), the alloantiserum was able to block the cytolytic reaction against the C57BL/6 anti-CBA $^{51}$Cr-blasts whereas the control anti-DBA/2 alloantiserum had no blocking ability. Again, these results indicate that there indeed exist cytolytic autoanti-idiotypic T cells in the spleens of autoblast immune mice.

*Transfer of Specific Suppression by Lymphocytes of Autoimmunized Mice into Normal Syngeneic Mice (3-9b in Experimental Plan).* To further analyze the mechanism of suppression of MLC in autoblast-immunized C57BL/6 mice, we carried out transfer experiments of cells from autoimmunized mice into normal, 100 rads-treated syngeneic recipients. These experiments are summarized in Table IV and we first tested if specific transfer of suppression was possible (no. 3 in experimental plan). $100 \times 10^6$ spleen cells from the pool of C57BL/6 mice autoimmunized with C57BL/6 anti-CBA MLC T lymphoblasts

## TABLE IV
### *Evidence for Idiotype-Specific T Suppressor Lymphocytes*

| Spleen cells from | Transferred cells treated with | Number of transferred cells injected into 100 rads irradiated mice | [³H]TdR incorporation of mixture with CBA stimulator cells | | [³H]TdR incorporation of mixture with DBA/2 stimulator cells | | [³H]TdR incorporation of responder cells alone | |
|---|---|---|---|---|---|---|---|---|
| | | | Day 4 | Day 5 | Day 4 | Day 5 | Day 4 | Day 5 |
| 1) Pool of three normal C57BL/6 | – | – | 206,889 ± 1,210 | 204,314 ± 4,777 | 206,471 ± 18,861 | 127,250 ± 5,473 | 2,547 ± 288 | 1,965 ± 32 |
| 2) C57BL/6 injected four times with Freund's adjuvant | Ig anti-Ig column | $3 \times 10^7$* | 201,905 ± 7,065 | 235,437 ± 12,764 | 210,428 ± 16,993 | 227,542 ± 25,777 | 3,064 ± 291 | 2,377 ± 107 |
| 3) C57BL/6 injected four times with $1 \times 10^7$ C57BL/6 anti-CBA MLC blasts | – | $1 \times 10^8$‡ | 58,217 ± 7,989 | 50,573 ± 5,697 | 189,586 ± 7,236 | 181,194 ± 14,606 | 3,233 ± 792 | 1,980 ± 121 |
| 4) " | Ig anti-Ig column | $3 \times 10^7$‡ | 40,691 ± 5,462 | 39,486 ± 4,638 | 198,934 ± 7,931 | 185,283 ± 5,789 | 4,666 ± 911 | 2,447 ± 560 |
| 5) " | anti-Thy-1.2 + C′ | $3 \times 10^7$‡ | 131,439 ± 4,412 | 125,592 ± 5,445 | 178,316 ± 8,053 | 174,391 ± 4,283 | 4,282 ± 290 | 2,430 ± 260 |
| 6) " | Ig anti-Ig + NMS + C′ | $3 \times 10^7$* | 52,233 ± 905 | 55,050 ± 3,685 | 191,399 ± 8,032 | 212,460 ± 11,719 | 4,251 ± 515 | 2,671 ± 510 |
| 7) " | Ig anti-Ig + anti-Ly-1.2 + C′ | $3 \times 10^7$* | 42,075 ± 1,980 | 43,460 ± 1,558 | 182,279 ± 8,611 | 181,764 ± 5,826 | 4,743 ± 95 | 2,847 ± 249 |
| 8) " | Ig anti-Ig + anti-Ly-2.2 + 3.2 + C′ | $3 \times 10^7$* | 111,830 ± 11,931 | 99,367 ± 17,534 | 193,148 ± 3,289 | 191,099 ± 6,372 | 4,404 ± 67 | 2,996 ± 175 |
| 9) " | Ig anti-Ig + C57BL/6 anti-CBA + Ig anti-Ig | $3 \times 10^7$* | 195,094 ± 7,964 | 213,497 ± 6,160 | 217,673 ± 15,818 | 183,673 ± 7,855 | 3,361 ± 340 | 1,827 ± 222 |
| | Ig anti-Ig + C57BL/6 anti-CBA + Ig anti-Ig cells eluted from column | $3 \times 10^7$§ | 20,601 ± 114 | 19,881 ± 3,735 | 188,333 ± 7,619 | 194,832 ± 12,310 | 3,581 ± 586 | 2,190 ± 395 |

1) Spleen cells of three normal C57BL/6 mice were pooled and used as responder cells in MLC against CBA and DBA/2 stimulator cells. This represents a positive control of normal untreated C57BL/6 mice. Figures denote mean ± SE of triplicate cultures.

2) C57BL/6 mice were injected four times with 0.2 ml of Freund's adjuvant in PBS in 3-wk intervals. Animals were killed 10 days after the last injection. Spleen cells were pooled and T cells purified over Ig anti-Ig column. $3 \times 10^7$ purified T lymphocytes were injected i.v. into 100 rads irradiated normal C57BL/6 mice. Animals were killed 1 mo later and the spleens individually tested in MLC against CBA and DBA/2 stimulator cells. Figures represent the mean ± SE of four animals which were tested in triplicate cultures.

3) C57BL/6 mice were injected four times in 3-wk intervals with $1 \times 10^7$ C57BL/6 anti-CBA MLC T lymphoblasts. Animals were killed 10 days after the last injection and the spleens pooled (see experimental plan). $1 \times 10^8$ spleen cells were injected into each of three normal 100 rads irradiated C57BL/6 mice. Animals were killed 1 mo later and further tested as described under 2).

4) The spleen cells derive from the autoimmunized pool (see 3). Cells were passed over Ig anti-Ig column and $3 \times 10^7$ purified T lymphocytes were injected into each of three mice (100 rads irradiated) which were tested 1 mo later as described under 2).

5) Spleen cells from the autoimmunized pool were treated with anti-Thy-1.2 and complement. $3 \times 10^7$ of the remaining B lymphocytes were injected into each of three mice which were tested 1 mo later as described under 2).

6) T lymphocytes from the autoimmunized pool were purified over Ig anti-Ig column and treated with NMS (normal mouse serum) and complement. $3 \times 10^7$ of the remaining cells were injected into each of four C57BL/6 mice. For further procedure, see 2).

7) T lymphocytes were purified as described under 6). Cells were treated with anti-Ly-1.2 and complement and $3 \times 10^7$ cells of the remaining population was injected into each of four C57BL/6 mice (further test see 2).

8) T lymphocytes were tested as in 7), but instead of using anti-Ly-1.2, anti-Ly-2.2, and anti-Ly-3.2 were used.

9) T lymphocytes were purified as under 7). Cells were incubated with C57BL/6 anti-CBA alloantiserum. Cells were passed over anti-Ig column and $3 \times 10^7$ of cells passing the column were injected into each of four C57BL/6 mice. Further testing see 2). Cells trapped in the column were eluted by stirring with a Pasteur pipette and $3 \times 10^7$ eluted cells were injected into each of two C57BL/6 mice. For further procedure see 2).

MLC was performed as described under Material and Methods in flat bottom microtiter plates. Triplicate cultures were pulsed for 6 h with 1 μCi of [³H]TdR.

* Mean ± SE of four mice which were individually tested in triplicate cultures.

‡ Mean ± SE of three individually tested mice. Each mouse tested in triplicate cultures.

§ Mean ± SE of two mice individually tested in triplicate cultures.

were injected intravenously into each of three normal 100 rads irradiated C57BL/6 mice. As a control, $10^8$ spleen cells from C57BL/6 mice immunized with Freund's adjuvant alone were injected. 1 mo later, animals were killed and their spleens analyzed in MLC against CBA or DBA/2 stimulator cells. As shown in Table IV, C57BL/6 mice injected with spleen cells from autoimmunized mice expressed a drastically reduced response against CBA stimulator cells but a close to normal response against DBA/2 stimulator cells. It is thus possible to transfer suppression by spleen cells. In a further experiment (no. 4 in experimental plan), the very same experiment was carried out as described above but instead of using spleen cells, purified T lymphocytes from autoimmunized animals were transferred. $3 \times 10^7$ Ig anti-Ig column purified C57BL/6 T lymphocytes from autoimmunized mice were injected into each of three mice. The response of these mice tested 1 mo later in MLC against CBA and DBA/2 stimulator cells was close to abolished against CBA alloantigens but virtually normal against DBA/2 stimulator cells. Thus, suppression can be transferred by immune T lymphocytes.

Suppressor as well as cytotoxic T cells belong to the Ly-2,3-positive T-cell subpopulation (13). We asked the question which subpopulation of T lymphocytes is able to transfer the MLC suppression into normal C57BL/6 mice. T lymphocytes from C57BL/6 mice autoimmunized with C57BL/6 anti-CBA MLC T lymphoblasts were incubated with either anti-Ly-1 or anti-Ly-2,3 antiserum and complement. The remaining cells were injected intravenously into normal, 100 rads irradiated C57BL/6 mice. Spleen cells of these mice were tested 1 mo later in MLC against CBA and DBA/2 stimulator cells. The results of the experiments are shown in Table IV (no. 7 and 8 in experimental plan). Ly 2,3-positive T lymphocytes and to a lesser extent Ly-1-positive lymphocytes were able to transfer unresponsiveness against CBA stimulator cells.

We now tested whether purified B lymphocytes from autoimmunized C57BL/6 mice also could transfer suppression of the MLC response against CBA alloantigens. Spleen cells from C57BL/6 mice autoimmunized C57BL/6 anti-CBA MLC T lymphoblasts were treated with anti-Thy-1.2 and complement before injecting $3 \times 10^7$ viable cells into the 100 rads irradiated normal C57BL/6 mice. Spleen cells from these mice were tested 1 mo later in MLC against CBA and DBA/2 stimulator cells. As depicted in Table IV, immune B lymphocytes are also able to transfer significant amounts of specific suppression. This partial effect is most likely not due to T lymphocyte contamination as analysis of the B lymphocyte preparation with FITC-labeled rabbit anti-mouse Ig indicated that at most, $6 \times 10^5$ T cells could have been transferred to each recipient. Quantitative titrations as to number of pure-immune T cells required have indicated a minimum figure of $10^7$ T cells to achieve significant suppression (H. Binz and H. Wigzell, unpublished observations). Whether a few helper cells could function in a more sensitive way is possible however. As a control, spleen cells from autoimmunized C57BL/6 mice were incubated with normal mouse serum and complement would affect the suppressive effect of the cells. As shown in Table IV (no. 6 in experimental plan), this is not the case.

Having established that the immune responsiveness could be transferred well by T cells and not as well, although significantly, by B lymphocytes from

the autoblast immune animals, we now carried out an experiment aiming to determine the antigen-binding specificity of the suppressor T cells (no. 9a and b in experimental plan). Earlier studies have taught us that idiotype-positive T cells can be rendered Ig positive by reaction with anti-idiotypic antibodies. Such Ig-coated T cells can then be selectively removed from other T cells via filtration through anti-Ig bead column and the retained cells can be recovered in a functionally intact form by using mechanical elution (12). In principle, it should be possible to do the very same thing with anti-idiotypic T cells, that is, incubating the present suppressor T cells from C57BL/6 mice immunized with autoblasts of anti-CBA specificity with conventional C57BL/6-anti-CBA alloantiserum followed by fractionation of the cells over a second anti-Ig column (the T cells were prepared by filtration over a first anti-Ig column before incubation with the alloantiserum). Passed and retained, eluted cells were then assayed for ability to transfer suppression by using the system as described before. As seen in Table IV, passed cells had entirely lost the ability to transfer suppression whereas the retained cells caused a close to complete suppression of the recipient mice indicating specific enrichment of relevant suppressor cells in the retained population. These latter data are thus strongly suggestive that the entire detectable suppressor ability detected at the T lymphocyte level is caused by true immune autoanti-idiotypic T cells.

## Discussion

The present article contains results enlightening the underlying cellular basis of the specific immune tolerance achieved by using autologous antigen-specific purified T lymphoblasts as the inducing agent. It had previously been found that such autoblast-immunized animals express specific reduction or complete elimination of immune reactivity towards the relevant alloantigens as visualized in MLC, CML, and graft-versus-host assays (1–3). Whereas the induction of specific suppression of the above T-cell functions was inducted in virtually all animals, although to a varying extent (1–3), only some animals produced detectable amounts of autoanti-idiotypic antibodies reactive with the receptors on the T blasts used for immunization (3). This could either be because our anti-idiotypic radioimmunoassay system was not sensitive enough, or alternatively T-cell-mediated suppressor functions may also exist in the blast immune animals. As the suppression of immune reactivity was selective only affecting reactivity against the expected histocompatibility antigens, only two possible T-cell functions for specificity were deemed possible: according to our first alternative, there existed autoanti-idiotypic T cells functioning in a specific suppressive manner against the idiotypic-positive, anti-alloantigen-reactive cells. A second alternative would be that soluble alloantigens derived from the original stimulator cells could be around on the surface of the MLC T blasts used for autoimmunization (8, 14) in such a way as to lead to induction of alloantigen-specific suppressor T cells.

It is now well recognized that autoanti-idiotypic immunity can be induced and shown to exist at both B and T lymphocyte levels (6, 9, 15–19). In the present autoblast immunization system we had already been able to demonstrate that spleen cells of such immunized animals could display a specific

cytolytic activity against syngeneic blasts of the right immunological reactivity (1). This cytolytic reactivity although anti-idiotypic as to its specificity was in these earlier experiments not analyzed as to effector cell type or mechanism. Thus, antibody-dependent cell-mediated cytolysis functioning via non-T effector cells and autoanti-idiotypic antibodies could equally well have been the underlying basis of the observed lysis as could true anti-idiotypic killer T cells. In the present article, we have shown that autoanti-idiotypic killer T cells can indeed occur as demonstrated by the use of purified T cells as effector cells and by using $^{51}$Cr-labeled T blasts of various genotypes and immune specificity as targets. The only cells lysed in these experiments were T blasts expected to carry the relevant idiotypic receptors (in this case C57BL/6 anti-CBA T blasts) whereas C57BL/6 anti-DBA/2 blasts were not lysed (signifies excluding auto-anti-blast killer cells in general) and neither were CBA anti-C57BL/6 blasts (signifies arguing against the reactivity being directed against hypothetical soluble CBA alloantigen still present on the C57BL/6 anti-CBA blasts). Furthermore, inhibition experiments of cytolysis with alloantisera produced in C57BL/6 against CBA were highly efficient inhibitors whereas other C57BL/6 alloantisera had no inhibitory power. In all, these data prove the case of the actual existence of autoanti-idiotypic killer T cells in the present system. Whether these cytolytic T cells recognize self-SD as well as idiotypic determinants in a manner analogous to e.g. killer cells against virus-infected cells (20), is an unknown but intriguing question. The system may, however, allow an analysis of inhibition of killer T-cell behavior in general as an inhibitor of soluble nature with highly specific activity was here demonstrable, namely the idiotype-positive alloantibody molecules.

A second new fact established in the present article that turned out to be very helpful in the analysis of the underlying mechanisms of specific immune unresponsiveness, the fact that this was possible to transfer to normal 100 rads irradiated syngeneic recipients. 100 rads was chosen as a radiation dose with comparatively little harmful effects on the immune capacity of the lymphoid tissue of the recipient (10) but a dose that would allow a highly significant increase in take of transferred syngeneic lymphoid cell (11). The fact that immune unresponsiveness as such can be transferred in certain systems to normal, syngeneic recipients is by far not new (21) but did here merely allow a more refined analysis as to which subgroups of cells were participating in the present autoblast system. T lymphocytes from blast-immunized animals were the most efficient cell type capable of transferring immune suppression in the present system. When analyzed as to Ly-phenotype, judged to be a useful marker to subdivide T cells into groups with large different functional ability (13), it could be shown that T cells with the Ly-$1^-2^+3^+$ markers were the most efficient suppressor cells. However, we already know that killer T cells exist in the present system with anti-idiotypic specificity and would thus stress that it is at present quite unclear whether the actual suppressive act is occurring via lytic or suppressive mechanisms. Cells with the surface markers Ly $1^+2^-3^-$ were significantly inferior although still able to induce significant suppression upon transfer. It is unlikely that this was caused by enough contaminating Ly $1^-2^+3^+$ in these populations as such cells were looked for by using sensitive

radioimmunoassays (9) after the cytolytic elimination of $2^+3^+$ cells but could not be demonstrated (data not shown). That helper cells (= Ly $1^+2^-3^-$ cells) could transfer some degree of suppression is not surprising, however, when realizing that autoanti-idiotypic antibodies of IgG type can be induced by autoblast immunization procedures (1). Such anti-idiotypic antibodies in presence of complement are quite able to selectively lyse away the idiotype-positive cells (1) and induction of antibody formation in recipient B cells via transferred-immune helper T cells could here play a significant role.

The finding that purified B lymphocytes from autoblast-immune animals also could transfer significant degrees of immunity cannot be explained by some simple degree of contamination with immune T lymphocytes. Thus, the highest possible number of contaminating T cells in these B cells transferred could only approach $^1/_{15}$th of the required number for purified T cells to transfer protection (data not shown). However, we do not know for sure whether the immune B cells alone upon transfer were able to produce enough additional autoanti-idiotypic antibodies to cause the detectable suppression. Alternatively, one may speculate that the possible tiny group of contaminating immune helper T cells plus the immune B cells together could have yielded more anti-idiotypic antibodies than completely pure, immune B cells. We deem this question, however, of comparatively little importance in the present context.

This article also contains a set of experiments which we consider quite decisive in showing that maybe all suppressive ability of the T lymphocytes in autoblast immune animals has autoanti-idiotypic specificity with no detectable, hypothetical alloantigen-specific suppressor cells. Thus, T cells from blast immune animals will bind enough idiotype-positive molecules as to allow a selective retention on anti-Ig columns with consequent enrichment of suppressor function. At the same time, the passed cells were depleted *in toto* of suppressive ability. These findings to our mind prove this point in a conclusive manner.

The present findings of an active, specific autoanti-idiotypic immunity being exerted by both B and T lymphocytes may well explain the long-lasting, specific-immune suppression observed in the autoblast-immunized animals. Here, it would thus seem likely that new viriginal idiotype-positive lymphocytes being continuously produced by stem cells may serve as an internal boostering device maybe allowing a life long state of specific unresponsiveness. It is obvious that such stable immune tolerance has both theoretical and practical implications.

## Summary

Specific immune unresponsiveness against a given set of histocompatibility antigens can be induced by immunization with autologous, antigen-specific T lymphoblasts. Such unresponsiveness can be transferred by lymphoid cells from autoblast-immunized donors to normal syngeneic recipients. The cells being most efficient in transferring the selective suppression are T lymphocytes from the spleen, especially if of Ly $1^-2^+3^+$ phenotype. By using such T lymphocytes we deem it likely that the actual underlying mechanism is one of actual transfer of autoanti-idiotypic killer T cells. In support for this view is

the fact that such T cells endowed with exquisite specific, cytolytic reactivity towards autologous idiotype-positive T target cells exist in autoblast immune animals. Significant suppression may also be transferred with T cells of Ly $1^+2^-3^-$ phenotype or with B cells. Here, we consider the suppressive mechanism to be one of production of autoanti-idiotypic antibodies. By using affinity fraction procedures, it was finally possible to prove that all T-cell suppressive activity resides in a population with true antigen-binding-specific receptors for the relevant idiotypes.

The expert technical assistance of B. Eisenegger is gratefully acknowledged.

*Received for publication 2 August 1977.*

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