IMMUNE STATUS OF MICE TOLERANT OF LIVING CELLS

II. CONTINUOUS PRESENCE AND NATURE OF FACILITATION-ENHANCING ANTIBODIES IN TOLERANT ANIMALS*

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It has been previously established in this laboratory that sera from CBA mice made tolerant to A/Jax skin grafts (neonatally induced tolerance) were able to enhance the growth of A/Jax tumors grafted on otherwise untreated CBA mice. This property was specifically removed by incubation with A cells (and not by CBA cells). The sera included those from highly tolerant mice, even taken before skin grafting, and the criteria for tolerance were severe, skin grafts in perfect state 100 days after grafting (1, 2).

These and other data demonstrated the state of immune reactivity of lymphoid tissue from tolerant animals toward tolerated cells (1-4) as well as the establishment of a state indistinguishable from that of immunological tolerance through immunological facilitation enhancement in the graft-versus-host situation (5-9). These results confirmed our views according to which immunological facilitation (the basis for the mechanisms of immunological enhancement) is also the basis for the mechanism of immunological tolerance of living cells (4, 10), implying an active immune reaction followed by an antibody mediation of some crucial step. Further confirmation came from converging evidence obtained in other laboratory experiments (11-18).

The purpose of the present work was to obtain some more precise data on the properties, time evolution, and nature of the antibodies responsible for the selective immuno-suppression that we believe leads to the state of immune tolerance to living cells (19). It consisted in studying the sera of highly tolerant mice as compared with those of corresponding normal and immune mice for their content in immunoglobulins of the different classes, in immuno-sero-biological activities, and in facilitation-enhancing antibodies (able to transfer tolerance to corresponding allografted tumors). Evolution of the two last types of properties was studied in mice followed from birth (and tolerance induction) to sacrifice (in a state of high tolerance), looking for modifications brought in the immune status by the time and by the allograft procedures. We also tried to elute specific antibodies from spleens of highly tolerant animals. Finally, sera (tol-
erant, immune, or normal) were fractionated on chromatography columns and the resulting fractions were studied for their content in immunoglobulins of the different classes, in immuno-sero-biological activities, and in facilitation-enhancing properties. Links between biological properties and immunoglobulin classes were looked for.

**Materials and Methods**

*Animals.*—CBA and A strain mice and their F₁ hybrids were bred in our laboratory as previously stated (1, 2, 9).

*A/Jax Tumors.*—Sarcoma I was obtained from Dr. I. Hilgert, Institute of Experimental Biology and Genetics, Praha, Czechoslovakia, and kept since 1969 in ascitic form in A strain mice. This sarcoma has proved in our laboratory to be more susceptible to enhancement in CBA strains than other SA substrains. On the other hand, it gives up to 33% of takes in normal CBA mice.

Sarcoma 1599la was obtained by courtesy of Dr. N. Kalis and kept in our laboratory since 1970 in A strain mice. It gives no take in normal CBA mice but is relatively less susceptible to enhancement.

*Induction and Evaluation of Tolerance.*—Tolerance to A strain tissue was induced in CBA newborn mice by intravenous injections of $15 \times 10^6 (CBA \times A) F₁$ spleen cells. For evaluation, mice were grafted at 8 wk with A skin (1, 2) and grafts were inspected weekly and recorded for their general aspect. Only grafts which did not present any rejection signs, thus closely resembling isografts, were kept for tolerant serum experiments. Each animal was numbered and followed individually. Mice having a perfect state of graft over 100 days were considered as highly tolerant.

*Serum Collection.*—Blood samples were drawn from the tail artery after heating the animals at $37°C$. A record indicating the animal’s number and time of blood collection was entered for each bleeding. Only samples from highly tolerant mice were pooled and used for the experiments presented.
Three pools were prepared (Fig. 1): I, before skin grafting; II, 2 wk after skin grafting and regular bleedings until day 80 after the graft; III, exsanguination at sacrifice, at the end of the 4th month.

**Extraction of Antibodies from Spleens of Tolerant Mice.**—Spleens were stored at −70°C, thawed at room temperature, and individually ground in a loose Potter-Elvehjem grinder in 0.5 ml of saline. The suspension was heated at 46°C for 30 min under continuous moderate shaking. The supernatant of a 5 min centrifugation (500 g at 46°C) was mixed with dextran (3 volumes of 6% intradex for 7 volumes of spleen extracts).

The preparations were tested against an equal volume (0.05 ml of a 2.5% suspension of A/Jax erythrocytes and left 1 hr at 37°C. CBA erythrocytes were used as control suspension.

**Fractionation of Pooled Sera.**—Three pools of CBA sera (10 ml each) were fractionated on diethylaminoethyl (DEAE)-cellulose columns: normal CBA sera, pool II from tolerant mice, and sera from mice immunized against A/Jax living spleen cells (five injections of $12 \times 10^8$ cells over a period of 2½ months).

Column chromatography was made on DEAE-cellulose with a continuous and linear gradient of NaCl (0.02-1 mM) in pH 8 tris(hydroxymethyl)aminomethane buffer, and 20 fractions were obtained (from slowest IgG's to albumin). Cold ethanol fractionation of serum pool III was performed according to Deutsch (20). The method was studied and modified to be applied to mouse sera, especially to fractionate immunoglobulins into slow and fast (electrophoretic) ones. It includes treatment with 25% cold (–10°C) ethanol and precipitations by pH adjustments at 7.7, 5.5, and 7.4 in succession giving rise to three sediments and three supernatants. The first and third supernatants, as well as the second and third sediments, are kept for analysis and study.

**Immunodiffusion in Gel Analysis of Fractions and Sera.**—Qualitative analysis was made through micro-Ouchterlony on cover slips (1% agar in 9% NaCl) and microimmunoelectrophoresis on histological slides (1% agar in Veronal buffer, 0.05 mM, pH 8.6). Monospecific (anti-IgM, anti-IgA, anti-IgG1, anti-IgG2) and plurispecific anti-mouse immunoglobulins and sera were used.

Semiquantitative analysis for immunoglobulin classes was made through Mancini's method of radial immunodiffusion, utilizing the same monospecific immune sera (21). The calibration was realized with pure normal IgG2 (without discrimination between IgG2a and IgG2b), with IgG1 and IgA from myeloma, and IgM from Sephadex G-200 first peak. The obtained figures are expressed in milligram per milliliter for sake of clarity, but knowing that they actually are expressions of relative values. For normal CBA serum they were found intermediate (as a whole) between those found by Fahey and Barth in C3H/He and C57BL/6NJ (22) and by Fahey and Sell (23) in NIH-WS mice (23).

**Immunosera-Biological Tests of Sera and their Fractions.**—The following tests were performed: hemagglutination according to Gorer and Mikulska (24); synergistic hemagglutination according to Gorer et al. (25), slightly modified (undiluted studied preparation is mixed with doubling dilutions of a standard CBA anti-A immune serum prediluted in such a way as to give three positive wells with A erythrocytes when mixed with control serum or fraction); cytotoxic activity, slightly modified from Chouroulinkov et al. (26); hemolytic activity, according to Hildemann (27); passive cutaneous anaphylaxis (PCA)1 according to Kinsky et al. (28).

**Evaluation of Facilitation-Enhancing Properties of Sera.**—6 $\times 10^6$ cells of SaI were injected subcutaneously into the right flank of each CBA male recipient. Recipients were injected intravenously the day before with 0.4 ml of serum. Two control groups received either normal CBA serum (NS) or CBA anti-A serum (IS) diluted 1/4. The latter was prepared in CBA animals

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1 Abbreviations used in this paper: NS, normal serum; PCA, passive cutaneous anaphylaxis; SaI, A/Jax sarcoma 1; Sa 15091a, A/Jax sarcoma 15091a.
which have received five injections of living A spleen cells within a period of 3 months. A strong enhancing activity was assessed for this serum in a preliminary trial.

Three groups of ten animals received serum pools I, II, and III, respectively. Mean tumor diameters were measured every 5 days and the day of the recipient’s death recorded. Mortality curves were plotted in probit units on a logarithm time scale giving almost straight lines. The rate of death was expressed by the slope of each line.

RESULTS

Properties of Sera from Highly Tolerant Mice (Compared with Normal and Immune Sera).—Sera were taken from the same CBA mice highly tolerant to A/Jax (see Materials and Methods and Fig. 1) at three different periods of tolerance: before skin graft test for tolerance (pool I), between days 14 and 80 after this test (pool II), and at time of sacrifice with a perfect graft (pool III) 120 days after grafting. All three were studied for serological and biological properties and compared with a pool of normal CBA sera and another pool of CBA immune sera anti-A/Jax.

Content in immunoglobulins of four classes: Semiquantitative analysis according to Mancini’s technique with monospecific anti-IgM, IgA, IgG1, and IgG2 (without distinguishing between G2a and G2b) allows an approximate value for the content in immunoglobulins of these four classes (Fig. 2). It must be first noticed that, as a whole the evaluated content of tolerant pool II in immunoglobulins is about 130% that of normal CBA serum pool, while immune CBA serum pool is 160% that of normal (normal 6.96 mg/ml, tolerant 9.00 mg/ml, and immune 11.24 mg/ml). The most striking feature is that the serum richest
in IgG1 is the tolerant one (3.10 mg/ml) followed by the immune serum (2.7 mg/ml) and by the normal one (2 mg/ml). For IgA, the tolerant serum concentrations are intermediate between that of immune and normal sera, being below the former and above the latter. Finally, complement-fixing immunoglobulins (IgM and IgG2) in tolerant sera are lower than in immune ones and comparable to those in normal sera.

**Serological activities:** The serological and PCA activities of the five sera (normal, immune, and the three tolerant pools) are simply summarized in Table I where it may be noticed that while immune serum had strong hemagglutinating, hemolytic, and anaphylactic activity and normal serum (as control) had no detected activity, the three pools from highly tolerant animals presented (as already described) synergistic hemagglutinating activity. This was less marked after skin graft (pool II), conceivably due to antibody absorption on the graft.

**In vivo facilitation-enhancing activity:** A/Jax Sal tumors grafted in CBA mice have a facilitated take and enhanced growth by treating recipients with sera either immune anti-A or tolerant of A (from any of the three pools) as compared with animals treated with normal CBA sera, showing the presence of facilitation-enhancing antibodies at various periods of high tolerance before and after skin grafting. The facilitating activity is shown both by the earlier deaths and by their increased proportion in recipients treated with immune or tolerant sera (Table II and Fig. 3).

Furthermore if the growth curves of the only lethal tumors are compared in the five groups (this can be done since there are some lethal takes even in the normal serum–treated controls), the following may be noticed (Fig. 4): mice injected with normal serum have a strong and early rejection reaction beginning after day 10, leading to an almost complete disappearance of palpable tumors,

| Origin of serum   | Direct hemagglutination | Synergistic hemagglutination | Hemolysis | Passive cutaneous anaphylaxis |
|------------------|-------------------------|-----------------------------|-----------|-----------------------------|
| CBA immune to A  | 260,000*                | N.D.‡                       | 66%§      | ++ +                        |
| Normal CBA       | —                       | —                           | —         | —                           |
| CBA tolerant of A|                         |                             |           |                             |
| Pool I (before grafting) | —                       | +3 dilutions               | —         | —                           |
| Pool II (14–80 days after grafting) | —                       | +1 dilution               | —         | —                           |
| Pool III (time of sacrifice)  | —                       | +4 dilutions               | —         | —                           |

* Reverse of titer.
‡ N.D. = not done.
§ Maximum percentage of hemolysis.
and ending around days 20–25; while animals injected with immune serum have a later, weaker, and shorter rejection reaction not leading to complete disappearance of palpable tumors, beginning after day 20 and ending around days 25–30. The situation is strikingly intermediate for animals injected with tolerant sera of the three pools.

### TABLE II

**Passive Enhancement Facilitation of Allografted SaI Cells with Sera from Highly Tolerant Animals**

| Origin of serum | Proportion of lethal tumors | Proportion of deaths on day 65* | Harmonic MST of grafted animals | Significance of difference to normal serum group |
|-----------------|-----------------------------|-------------------------------|---------------------------------|-----------------------------------------------|
| Normal CBA      | 4/10                        | 0/10                          | 197                             | (P < 0.02)                                   |
| CBA tolerant of A |                             |                               |                                 |                                               |
| Pool I (before grafting) | 5/5 (P < 0.05) | 1/5 (NS) | 73 (P < 0.02) | (P < 0.05) |
| Pool II (14–40 days after grafting) | 9/10 (P < 0.05) | 6/10 (P < 0.01) | 60 (P < 0.01) | (P < 0.01) |
| Pool III (time of sacrifice) | 9/10 (P < 0.05) | 7/10 (P < 0.005) | 56 (P < 0.02) | (P < 0.02) |
| CBA immune to A  | 4/5 (NS)                    | 4/5 (P < 0.005)               | 54 (P < 0.02)                   | (P < 0.02)                                   |

* All control animals surviving on that day. SaI cells are injected in CBA mice having received CBA serum from mice either normal, tolerant of A, or immune to A. They are followed for their survival time.

**MST = mean survival time.**

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**Fig. 3.** Cumulative mortality curves of CBA mice grafted with A/Jax sarcoma SaI and injected with sera from CBA mice (either normal, immune, or tolerant). Prob-log scales (percentages of mortality in probit units, survival time in logarithms of days), allowing drawing of mortality curves in straight lines. The onsets and the slopes of the curves, as well as the final death percentages, deserve attention. **CG** = center of gravity of the curves (points of coordinates x, y). (x) indicates a point 0% (pool I) that would, in probit scale, be placed at the infinite position on the curve.
Finally, when comparing the growth curves on nonlethal (finally rejected) tumors in the various groups, the following may be seen: in normal serum-treated mice initial growths occurred up to day 10, reaching 10 mm in diameter with complete regression (no palpable tumor) after day 15. In immune serum-treated animals, the only survivor had a tumor of maximum size (19 mm) on days 15–20 with complete regression after day 45. In tolerant sera-treated animals (pools II and III, no survivors with pool I), the two survivors had tumors of maximum diameter (10–12 mm) on day 10 with complete regression on days 20–25 (that is 5–10 days later than with normal serum). Thus, even in surviving animals, a difference was observed indicating a facilitation-enhancing action of immune and of tolerant sera.

No significant difference has been found between the facilitating activities of the three pools of tolerant sera: pool I (pregraft), II (postgraft), and III (exsanguination). The centers of gravity of the cumulative mortality curves of Fig. 3 (points with coordinates equal to the means of the experimental points of the same curve) are strikingly close for the three pools.

Specificity of facilitating activity in tolerant sera: Enhancing activity was tested in Sa 15091a grafted on CBA mice. This tumor proved in previous trials to be highly specific for A strain (with 55 rejections in 20 days out of 55 CBA mice injected with 4–6 million tumor cells). Three aliquots of 5 ml of the same tolerant serum (CBA tolerant of A) were either left untreated or absorbed 30 min at 37°C with 1 ml of packed sarcoma cells or “pseudoabsorbed” in similar conditions with CBA spleen cells. They were passed through Sephadex...
G-200 column (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) and the concentrated immunoglobulin-containing fractions (as well as similar fractions from normal CBA serum) were injected into CBA mice grafted 24 hr later with Sa 1509Ia. In 17 unabsorbed tolerant serum fractions-treated animals, two lethal takes were observed, as in 2 of 18 animals treated with pseudoabsorbed tolerant serum fractions; complete rejections were observed in all of 18 mice injected with specifically absorbed tolerant serum fractions as well as in 16 mice injected with similar fractions from normal CBA serum. The over-all results were therefore 11% lethal takes in 35 mice injected with unabsorbed or pseudoabsorbed tolerant serum fractions vs. 0% in 89 mice (18 injected with absorbed tolerant fractions and 71 injected with either normal CBA serum or normal CBA fractions). The difference between these two groups is significant ($P < 0.01$).

| Immune status of CBA mice | Total No. of mice | No. of mice with specific hemagglutinating activity and its degree* |
|---------------------------|------------------|---------------------------------------------------------------|
|                           |                  | Negative | Positive |     |     |     |
| Tolerant of A             | 59               | 47 (80%) | 4        | 3   | 4   | 1   |
| Normal                    | 20               | 20       | 0        | 0   | 0   | 0   |
| Immune to A               | 5                | 0        | 0        | 0   | 0   | 5   |

* Spleen extracts of these CBA mice were tested against A/Jax erythrocytes for hemagglutinating activity. CBA erythrocytes were used as negative controls.

**Extraction of Antibodies from Spleens of Highly Tolerant Mice Compared with Normal and Immune Mice.**—59 spleens from exsanguinated, highly tolerant mice, taken at day 120 of tolerance, were individually treated by procedures of concentrated extraction and of moderate heat elution (see Materials and Methods). They yielded 59 preparations, 20% of which were able to give some degree of direct hemagglutination (Table III). Although no absorption experiments could be performed, negative controls were represented, by spleens from normal CBA mice, and positive controls by spleens taken since day 5 after immunization of CBA mice against A/Jax by means of either spleen cells or skin grafts. Furthermore, all these spleen extracts (from CBA mice either normal, tolerant of A or immune to A) were negative when tested against CBA mice erythrocytes. Therefore, while direct hemagglutinating antibodies were not found in sera of these highly CBA tolerant mice, some could be extracted from one out of five of the spleens of the same animals.

**Properties of Chromatographic Fractions from Sera of Highly Tolerant Mice (Compared with Normal and Immune Sera).**—Three pools of sera were chosen
for fractionation among the five that were studied above for their various activities: immune serum pool, normal serum pool, and tolerant serum pool II (the most abundant).

Immune and normal pooled sera gave the expected elution profiles (Fig. 5) and immunoglobulin classes distribution (Fig. 6). Also expected was the distribution of serological activities of immune fractions: complement-fixing antibodies (IgG2) first eluted and hemagglutinating antibodies eluted on a broad range of molarities. Furthermore anaphylactic activity (passive cutaneous anaphylaxis) was found mainly in fractions 4 to 11, as analyzed elsewhere (28).

As expected, no activity whatsoever was found in fractions of normal sera thus insuring that activities eventually found in fractions of tolerant sera were not artifactual, even if not found in the whole sera before fractionation.

In tolerant serum pool II, the findings were as follows:

_Elution profiles and immunoglobulin contents (of the four classes):_ The elution profile was not different from those of the normal and immune sera (Fig. 5). The immunoglobulin content of the fractions, however, was different (Fig. 6): the IgG2 profile was as moderate as the one for normal serum while it dominated the profile of the immune serum in every single fraction (with a rebound of fast anionic IgG2, which later proved to be IgG3b). IgG1, however, dominated most of the profile of the tolerant serum, except for the less anionic part (with IgG2 predominating in the first four fractions) and the most anionic part where IgA
Fig. 6. Immunoglobulin content of CBA sera fractions from immune, tolerant, or normal mice.

Fig. 7. Immunoglobulin content (of four classes), synergistic hemagglutination, and facilitation-enhancing properties of tolerant sera DEAE fractions.
dominated. In contradistinction, IgG1 did not dominate in normal and immune sera, and their profiles were rather similar to each other. This was, to a lesser extent, also the case for IgA. Nothing remarkable was seen for IgM except that it was less prominent in tolerant than in immune fractions.

**Immuno-serological activities of fractions (Fig. 5):** Two properties, absent in the unfractionated tolerant sera, appeared: a weak specific immune hemolysis (<20%) in two cationic fractions of IgG2 and a weak direct hemagglutination in IgG2 + IgG1 fractions. Synergistic hemagglutination, detected in unfractionated serum, was localized in fast anionic globulins with a curve of intensity paralleled by the one of IgA concentration (Fig. 7). Tolerant fractions gave no anaphylactic activity except for a limited one at the peak of IgG1 concentration.

**TABLE IV**

**Biological Activities of Antibodies in Cold Ethanol Fractions of Pool III Tolerant Sera**  
(CBA Tolerant of A)

| Fractions                  | Predominant immunoglobulin class | HA activity | HL activity | PCA activity | Facilitating- enhancing activity |
|---------------------------|---------------------------------|-------------|-------------|--------------|----------------------------------|
| First supernatant (pH 7.7) | IgG1 (as contaminant, fraction essentially albuminic and α-globulinic) | 0           | 0           | +            | -                               |
| Second sediment (pH 5.5)  | Mixed (mainly β-globulins)      | 64          | 0           | -            | -                               |
| Third supernatant (pH 7.4) | IgG1                             | 16          | 24          | +            | +                               |
| Third sediment (pH 7.4)   | IgG2                             | 32          | 30          | -            | -                               |

HA = Hemagglutination (reverse of titer); HL = hemolysis (maximum percentage of hemolysis).

**Facilitating activity:** This was tested by looking for enhanced growth of Sa 15091a grafted in CBA mice injected with serum fraction. This sarcoma presents no take in normal CBA mice but is less susceptible to enhancement than SaI. A moderate but definite action was found by comparing the tumor growth. This facilitating action was found linked to synergic hemagglutination activity of the fractions, as well as to the presence of IgA (P < 0.001), and not to that of IgG2, IgG1, or IgM (Fig. 7). This is at variance with what is usually found in immune sera where the facilitating action has been found in close agreement with anaphylactic IgG1 antibodies (these experiments and reference 28).

**Cold ethanol fractionation of pool III:** Tolerant sera, pool III, were fractionated according to Deutsch's technique and examined for their content in immunoglobulins and serological activity. It has been found (Table IV) that IgG1-containing fractions had PCA activity, IgG2-containing fractions had
hemolytic activity, and that the second sediment (at pH 5.0) devoid of both PCA and hemolytic activity still had a clear hemagglutinating activity (1/64). Facilitation-enhancing activity on SaI tumor growth on CBA recipients was detected in IgG1-rich third supernatant that also presented a moderate hemagglutinating activity and some degree of hemolytic activity. The specificity of these serological reactions was controlled as follows: no activity whatsoever was found on CBA erythrocytes and local increased vascular permeability (PCA) was only observed at the time when, and only if, A/Jax antigen is injected intravenously.

**DISCUSSION**

The main elements brought out by these experiments which will be discussed are the following: the continuous presence of facilitation-enhancing antibodies in sera from tolerant animals; the character of high tolerance of all animals of which the sera were studied; the presence in fractions and extracts of immune activities absent in whole sera from tolerant animals; the particular immunoglobulin class profile of tolerant sera as compared with normal and immune ones; the double link between synergic hemagglutination, IgA immunoglobulins, and facilitating properties. These elements raise several problems, such as the nature of the responsible antibodies and the relations between tolerance and facilitation.

**Continuous Presence of Facilitating Antibodies in the Sera of Tolerant Animals.**—The presence of facilitation-enhancing antibodies in the sera of tolerant animals had already been described and their specificity demonstrated (1, 2), but their evolution has been followed here from 28 days after birth to almost 6 months (time of sacrifice) by means of the three pools of sera: pool I (pretest graft), pool II (posttest graft), and pool III (sacrifice). Facilitating antibodies, as well as antibodies responsible for synergic hemagglutination, have been seen to be continuously present. No striking differences were found in the properties of the three pools. Small differences were still noticed such as a depression in synergic hemagglutinins in pool II as compared with I and III (Table I). This might be due to fixation on skin graft antigens. Also the enhancing activity for tumor growth was almost identical for pools II and III, while in pool I it manifested itself later, more rapidly, and more completely (100% lethal growth; Fig. 3). Finally, the properties of pool III looked in several circumstances relatively closer to those of immune sera than did the other two pools, for instance, in the growth kinetics of tumors enhanced by pool III (Fig. 4) or by the presence of hemolysins in cold ethanol fractions of pool III. The specificity of these facilitating antibodies, already demonstrated (1, 2), have been confirmed again by absorption experiments. The continuous presence of these antibodies in the animals studied excludes the possibility that they would represent the beginning of an interruption of tolerance. This is even more strongly excluded by the following section.
Character of High Tolerance of all Animals Studied.—Only sera from highly tolerant animals entered the three pools. The criteria for high tolerance were most severe: perfect state of skin graft (looking like isografts) during the life of the animals, including the day of sacrifice. These criteria were already met in the preceding experiments (1, 2). Another criterion of high tolerance is also fulfilled in these experiments: total absence of circulating antibodies detected in the sera by classical tests of hemagglutination, cytotoxicity, and hemolysis (direct hemagglutinins were sometimes found during preceding experiments in sera from otherwise highly tolerant mice [1, 2]). The very demonstration of facilitating antibodies in the sera of highly tolerant animals shows an actual transfer of tolerance towards the corresponding antigenic cells.

Presence in Fractions and Extracts of Immune Activities that are Absent in Whole Tolerant Sera.—While direct anti-A hemagglutinins were not found in whole unfractionated sera of the highly tolerant animals, they were found in three preparations made from these animals: in 20% of the spleen eluates, in DEAE fractions, and in cold ethanol fractions (in both cases in IgG1- and IgG2-containing fractions). Even a small amount of hemolysis was found in IgG2-containing DEAE fractions of pool II tolerant sera and cold ethanol fractions of pool III tolerant sera. The cause of the absence of expression of these antibodies in unfractionated sera is not some nonspecific inhibitor as is shown by the fact that mixing equal volumes of positive fractions with whole tolerant serum led not to inhibition but to an increased hemagglutination titer (synergic action). The possibility remains, but has not been explored, that different fractionated classes of antibody exert a competitive action.

Particular Immunoglobulin Class Profile of Tolerant Sera as Compared with Normal Ones and with Immune Ones.—Schematically, tolerant sera are close to immune ones for noncomplement-fixing immunoglobulins IgG1 and IgA, while for complement-fixing immunoglobulins IgM and IgG2 they are close to non-immune, normal sera. Indeed pool II, studied for its content in immunoglobulins, was found to contain 155% as much IgG1 and 189% IgA, but only 95% IgM and 107% IgG2 as pooled serum from normal mice. This elevation of the serum content in immunoglobulins of certain classes is taken as an indication that specific antibodies of the same classes are probably increased, while antibodies of unelevated classes would not be increased. This is in keeping with views discussed further below on the relations between tolerance and facilitation and on the nature of responsible antibodies.

Link between Synergistic Hemagglutinins, IgA, and Facilitating Antibodies.—Synergistic hemagglutinins (antibodies unable to cause direct hemagglutination but increasing the hemagglutinating titer of a reference immune serum) have been detected in more than two-thirds of tolerant sera (1, 2). They are again found in the three pools of tolerant sera. Synergistic hemagglutination seems to be a special manifestation of antibody activity, possibly due to a particular immunoglobulin. Indeed, a link between synergistic hemagglutinins and the
presence of IgA, already suggested in electrophoretic fractions of immune IC anti-A serum (29), was demonstrated \( P < 0.001 \) in DEAE chromatography fractions of pool III tolerant sera (CBA tolerant of A) (Fig. 7). Further links were suggested in certain cases between facilitation-enhancing properties and the presence of IgA as well as synergistic hemagglutinins in fractions of immune sera (29, 30). Finally a link between tolerance and the presence of synergistic hemagglutinins was suggested (1, 2). The earlier findings and suggestions were confirmed and correlated by the present description of a highly significant link between the presence of IgA, synergistic hemagglutinins, and facilitating properties in fractions of sera from highly tolerant mice (Fig. 7). This makes conceivable the possibility that antibodies of the IgA class responsible for synergistic hemagglutination would play a role in the mechanism of tolerance of living cells.

Facilitating antibodies responsible for tumor growth present in transplantation immune sera have however been found linked to IgG1 and anaphylactic properties in the same CBA-A combination (31), although a link with IgA and synergistic hemagglutination was suggested in the IC-A combination. In any event, the responsible antibodies were localized in the electrophoretically fast migrating (29, 30) and DEAE gradient nonearly eluted fractions (31) also characterized by their content in noncomplement-fixing antibodies. Electrophoretically slow migrating antibodies were found to be rather inhibitory for tumor grafts (except when sufficiently diluted). These results were also in agreement with similar results by Chard (32) and Chard et al. (33). The main discrepancy is with the authors who find the facilitation-enhancing activity localized in complement-fixing, cytotoxic IgG2 antibodies (34, 35). Incidentally, enhancing properties have several times been seen in this laboratory to occur also in fractions apparently containing only IgG2 (especially IgG2a) antibodies, especially when they are diluted. One must therefore consider that either different parts of the phenomenon (such as central immunodeviation vs. peripheral blockade) are due to different immunoglobulins (36), that the responsible antibodies belong to another class of immunoglobulin which is not similarly fractionated in the different systems, or that several immunoglobulins may play a role when they are in certain proportions, as suggested by Linscott's experiments (37) for peripheral protection. These proportions would be obtained in different fractions for different systems.

**Relation between Tolerance and Facilitation Enhancement.**—It is interesting that antibodies, especially of a particular type (synergistic hemagglutinins), are present in sera of tolerant mice. It is more interesting that they are continuously present in the sera of highly tolerant animals and that they are related to the state of tolerance (see above and Voisin et al. [1, 2]) and not due to a state of incomplete tolerance or due to a beginning interruption of tolerance. But it is even more interesting that they have facilitating properties, allowing the take of homografts of tumor cells otherwise untolerated and rejected. This suggests
that the phenomenon is an active immunological response (of a special type related to the facilitation-enhancement phenomenon). This hypothesis has already been put forward (1, 2, 16, 17) and experimental support has been brought to it by the following facts: (a) Active and passive procedures of facilitation may lead to a state of specific depression of immune reactivity indistinguishable from that of immunological tolerance in the graft-versus-host situation (5, 7, 9) and in immune reactions towards sheep red blood cells (16, 17). (b) Lymphoid tissues of animals tolerant of allogeneic cells are in a state of reactivity closer to that of the immune state than of normal animals as shown for pyroninophilia, immunoglobulin synthesis, and specific graft-versus-host reactivity in some combinations (3, 10). (c) Specific antibodies exist in highly tolerant animal sera such as synergistic hemagglutinins. Some are fixed on presumably tolerated cells as shown by immunofluorescence and hemagglutination (1, 2). (d) Passive transfer of tolerance can be realized by serum from highly tolerant animals provided one utilizes a sensitive system (1, 2, 25, and this paper). Work in other laboratories has also substantiated the preceding hypothesis by showing that states of tolerance can be adoptively transferred (38-40), that antibodies may help or allow the in vitro induction of tolerance (11, 41), and that the presence of sensitized cells, as well as counteracting serological properties, inhibit the destructive action of cells in animals made tolerant at birth (14), after irradiation repopulation (13), or even in allophenic mice (18).

If immune tolerance results from an active immune response, one may wonder about the mechanism and the reasons for the usually observed differences between classical facilitation (with circulating antibodies) and classical tolerance (without circulating antibodies), as well as the inertia of lymphoid cells from tolerant animals in graft-versus-host or mixed lymphocyte culture situations (42, 43). It is not inconceivable and in keeping with the present results, as well as others (1, 2, 4, 6, 44-47), that "immunodeviation" (split tolerance) is the intermediary step between classical enhancement facilitation and classical tolerance. It combines the depression of both cellular hypersensitivity and complement-fixing antibodies with conservation or elevation of noncomplement-fixing antibodies. Even the later could be progressively depressed due to lack of helper cells as discussed elsewhere (19).

SUMMARY

CBA mice were rendered highly tolerant to A/Jax cells by neonatal intravenous injections of (CBA × A)F1 spleen cells. The high degree of tolerance was ascertained by the absence of circulating antibodies detected in the sera by the usual tests and by the perfect state of A skin grafts during all the experiments. Tolerant sera (sera from tolerant animals) were studied at three periods of tolerance: before skin test grafting, from 2 to 11 wk after grafting, and at time of sacrifice at almost 6 months of age.

The tolerant sera were shown to have specific facilitation-enhancing proper-
ties promoting the take and growth of A/Jax sarcoma (SaI and /Sa 15091a grafted on normal CBA mice. These properties were present throughout the duration of the experiments, showing that they were not the result of a beginning interruption of tolerance. The tolerant sera, although lacking the usual serological properties (hemagglutination, hemolysis, cytotoxicity, passive cutaneous anaphylaxis) had, however, specific synergistic hemagglutinating properties (increasing the hemagglutinating titer of a reference immune serum). Antibodies giving direct specific hemagglutination could be extracted from spleens of 20% of highly tolerant mice. The tolerant sera were also found to contain more IgG1 and more IgA than normal sera while they contained normal quantities of the complement-fixing immunoglobulins IgG2 and IgM.

Fractionation of tolerant sera on DEAE chromatography column confirmed the data concerning immunoglobulin classes and demonstrated direct specific serological activities undetected in unfractionated sera: a weak hemolysis in the most cationic fractions and a weak hemagglutination in the middle fractions. Synergistic hemagglutination, detected in unfractionated serum, was localized in fast anionic fractions containing high IgA concentration, along with facilitation-enhancing activity, thus confirming a link suggested previously between these three properties.

The relation between immunological tolerance and facilitating antibodies was discussed in the light of the fact that antibodies, possibly of a particular class continuously present at low dose in the sera of highly tolerant animals, are able to transfer (at least partly) this state of tolerance provided a sensitive test system is utilized.

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