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Quantitative and Qualitative Analysis of the Induction of Specific Immunologic Unresponsiveness in Adult Mice

By

Shigeru MURAMATSU and Susumu KAWAGUCHI

The Laboratory of Radiation Biology, Department of Zoology, Faculty of Science, Kyoto University, Kyoto, Japan

ABSTRACT Nonaggregated solution of a purified fraction of bovine γ-globulin was not immunogenic but induced the specific immunologic unresponsiveness. No significant difference was found between normal and x-irradiated mice with respect to the amount of antigen required for inducing the unresponsiveness. The same antigen incorporated into Freund's adjuvant was highly immunogenic to interfere with the immunosuppressive action of the nonaggregated antigen. The physical form of antigen and the concentration in the extracellular space seem much more important factors than the population of the immunologically competent cells in the induction of the unresponsiveness.

Introduction

We have much information about the relationship between the immunologic capacity of animals and the dose of antigen required for inducing the specific immunologic unresponsiveness (1-4, 8, 10-13, 16, 17). The large part of the information has revealed that the immunologically immature or inadequate animals, such as newborns, irradiated or radiomimetics-treated adult animals, can be rendered unresponsive by a smaller amount of antigen than that required for normal adult animals. To make the normal adult animals unresponsive, a large amount of antigen has often been employed, exceeding the appropriate amount for eliciting the antibody response by many factors (5, 9). Dresser (7) reported, however, that the nonaggregated bovine γ-globulin was not immunogenic but was able to induce the unresponsiveness (paralysis) in adult mice by very small amounts of microgram order. Since the nonimmunogenic form of antigen may persist without the rapid immune elimination even in normal adult animals, the employment of such antigens instead of immunogenic forms makes it possible to compare directly the immunosuppressive efficiency of antigens

in normal animals with that in immunologically inadequate animals having a smaller population of immunologically competent cells.

Any significant difference in the amount of the nonimmunogenic form of antigen required for establishing the specific unresponsiveness was not found between normal and x-irradiated mice which were employed in the present experiment as a representative of the immunologically inadequate animals. Some quantitative difference was suggested to exist between the immunogenic information and the immunosuppressive information for the immunologically competent cells, since the suppressive effect of nonimmunogenic form of antigen was counteracted by the additional administration of the immunogenic form of the same antigen.

Materials and Methods

Animals. Ten-weeks-old female ddS mice supplied from the Central Animal Laboratory of School of Medicine, Kyoto University, weighing about 25 g were used.

Antigen. Bovine γ-globulin (BGG) (Cohn Fraction II, Armour Pharmaceutical Co., lot no. X30604) was fractionated by passage through a dimethylaminoethyl cellulose column and a fraction (FI-BGG) eluted with 0.008 M tris-phosphate buffer pH 8.0, followed by dialysis and lyophilization, was used as the test antigen. This FI-BGG was shown by immunoelectrophoresis to mainly consist of γ_2 component, while the raw sample contained several other serum proteins.

Induction of specific unresponsiveness. One per cent FI-BGG in saline was centrifuged at 30,000 x G for 60 min. to obtain the nonaggregated soluble protein (sBGG) in the supernatant fluid. Mice were injected intraperitoneally with 0.5 ml of the saline solution containing given amounts of sBGG.

Challenge or immunization. The 1:1 mixture of the saline solution of FI-BGG and the Freund's incomplete adjuvant, made up with 4 volumes of Bayol 55 (Esso Standard Oil Co.) and 1 volume of Arlacel A (Atlas Chemical Industries, Inc.) was converted into a water-in-oil emulsion (Adj-BGG) with a laboratory mixer. Mice received 0.3 ml of Adj-BGG (routinely contained 2 mg of FI-BGG) by subcutaneous injection. Challenge injection was performed 20 days after the injection of sBGG.

Determination of immune or unresponsive state. FI-BGG was trace-labeled with ^{131}I (I*) (carrier free NaI*, Dainabot Lab.) by the method of Dresser (6) as previously described (15). About 0.5 μc of I* was finally tagged to the 2 mg of the protein (I*-BGG). Each mouse received 2 mg of the nonaggregated I*-BGG intravenously 4 days after the challenge or immunization with Adj-BGG. Bleeding of about 0.05 ml each was performed via the orbital plexus. The first bleeding was done shortly after the injection of I*-BGG to estimate the total volume of the blood. Mice were bled thereafter at 2-day-intervals.

The amount of blood in each bleeding was determined by the increment of the weigh of the glass tube. The radioacitvitv was measured with a well-type γ-ray scintillation counter (Nuclear Chicago). The counts were corrected for the decay of I* and for the dilution of I*-BGG in the circulation due to bleeding. Mice were given drinking water containing 0.2% KI starting 2 days before the injection of I*-BGG and thereafter.

In a part of the experiment, mice were bled by heart puncture 15 days after the challenge, and the anti-BGG antibody titers of their sera were determined by the passive hemagglutination technique using tanned sheep red cells sensitized with FI-BGG, according principally to the method of Stavitsky (19).

Irradiation. Whole body x-irradiation was performed with a Philips 250 kv machine. Mice were placed in perforated polystylene boxes on a turn table, and irradiated at 200 kv and 19 ma, target distance 55 cm, with a filter of 0.5 mm Cu and 1.0 mm Al, at the rate of 50 R / min.

Results

The preliminary test on sBGG, Adj-BGG and x-ray. Thirty mice were divided into 6 groups of 5 mice each. Mice in groups 1 and 2 were not irradiated. Groups 3 and 4 were irradiated with 200 R X-ray and groups 5 and 6 were irradiated wiih 300 R. On the following day, groups 1, 3 and 5 were immunized with Adj-BGG, and others were left untreated. Four days later, all mice were injected with 2 mg of nonaggregated I*-BGG intravenously and its elimination from the circulation was traced. The results indicated that firstly, Adj-BGG was highly immunogenic resulting in the rapid elimination of I*-BGG, secondly, I*-BGG injected intravenously was not immunogenic at least during the period under observation, and thirdly, x-ray did not influence the non-immune elimination of I*-BGG but delayed the immune response of mice injected with Adj-BGG (Fig. 1). This may mean that the x-ray reduces the population of immunologically competent cells, and that the retention of sBGG is independent of x-irradiation.

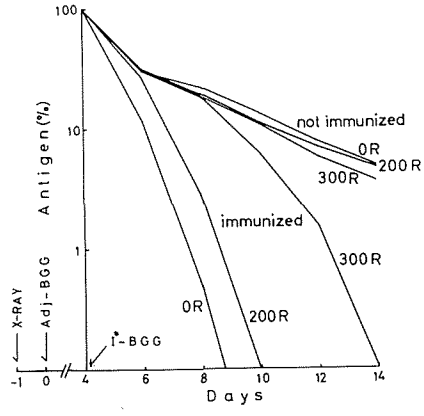

Fig. 1. The effect of x-irradiation on the elimination of I-BGG from the circulation of immunized and non-immunized mice. Each line represents the average of five mice.

Induction of the specific unresponsiveness by sBGG. Mice were injected with varying amounts of sBGG intraperitoneally, followed by the challenge with Adj-BGG 20 days later. The patterns of the elimination of I*-BGG administered 4

days after the challenge were classified arbitrarily into four categories of immunologic unresponsiveness as schematically represented in Figure 2; the area between the line of natural clearance (injected only with I*-BGG) and that of

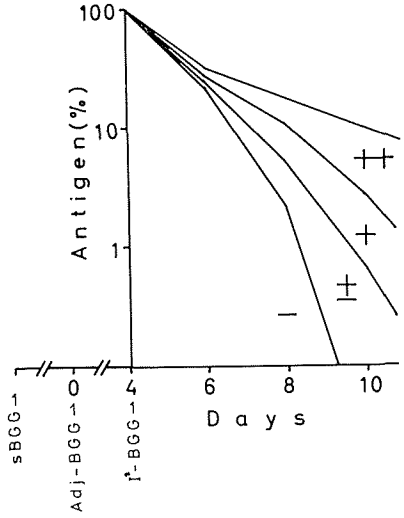

Fig. 2. Schematic representation of the category of immunologic unresponsiveness. The area between the elimination line of non-immunized mice (given only I*-BGG) and that of immunized mice (not given sBGG) is divided intn three parts. The categories are designated in decreasing order of the degree of unresponsiveness as ++, +. ±, and −.

Table 1

The induction of the specific immunologic unresponsiveness by sBGG[a] administered 20 days before the challenge with Adj-BGG[b]

sBGG (μg)	Category of Unresponsiveness				Log_2 Anti-BGG Titer[c]			
	++	+	±	−	≦ 2	3, 4	5, 6	7 ≦
1000	2/5[d]	3/5	-	-	2/5	3/5	-	-
100	5/6	-	1/6	-	5/6	-	-	1/6
10	1/5	1/5	3/5	-	1/4[e]	-	-	3/4
1	1/4	1/4	-	2/4	1/4	-	1/4	2/4
0.1	1/5	1/5	-	3/5	1/5	-	2/5	2/5
0	-	-	-	4/4	-	-	-	4/4
0[f]	4/4	-	-	-	4/4	-	-	-

a Dissolved in physiological saline and injected intraperitoneally.
b 0.3 ml of the emulsion containing 2 mg of FI-BGG was injected subcutaneously.
c Determined 15 days after the challenge.
d Number of mice per total number in each experimental group.
e One mouse died 12 days after the challenge.
f Not challenged with Adj-BGG.

immune elimination (untreated with sBGG) was divided into three parts, referring to as ++, +, and ±. Fifteen days after the challenge injection of Adj-BGG, all mice were bled by heart puncture and anti-BGG in their sera were titrated by means of passive hemagglutination technique. The results in Table 1 showed that sBGG was available for establishing the state of specific unresponsiveness, especially at the dose of 100 μg or more. This was realized in the categories of unresponsiveness in parallel with the anti-BGG hemagglutination titer.

The comparison of the effect of sBGG between normal and x-irradiated mice. Mice were divided into 3 groups, one of which was not irradiated and two others were irradiated either with 200 R or with 300 R. Each group comprised 7 subgroups, of which five were injected intraperitoneally with varying amounts of sBGG 24 hours later and other two (nonimmune and immune subgroups) were left untreated. The challenge injection with Adj-BGG and the injection of I*-BGG were performed according to the standard schedule. The patterns of the elimination of I*-BGG from the circulation of these mice are illustrated in Figure 3. Figure 4 shows the result of a experiment in which a part of the

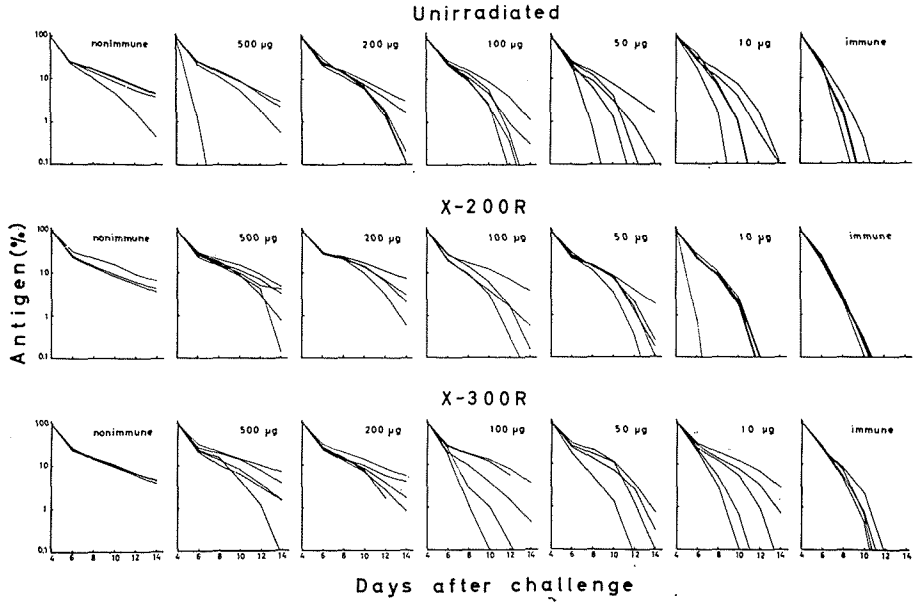

Fig. 3. The elimination of I*-BGG from the circulation of unirradiated and x-irradiated mice. The mice were injected with sBGG at the doses shown in each graph 24 hours after irradiation and were challenged with Adj-BGG 20 days later. I*-BGG was injected 4 days after the challenge. Each line represents the elimination of I*-BGG from one mounse. Nonimmune mice were not injected either with sBGG or with Adj-BGG, and immune mice were not injected with sBGG.

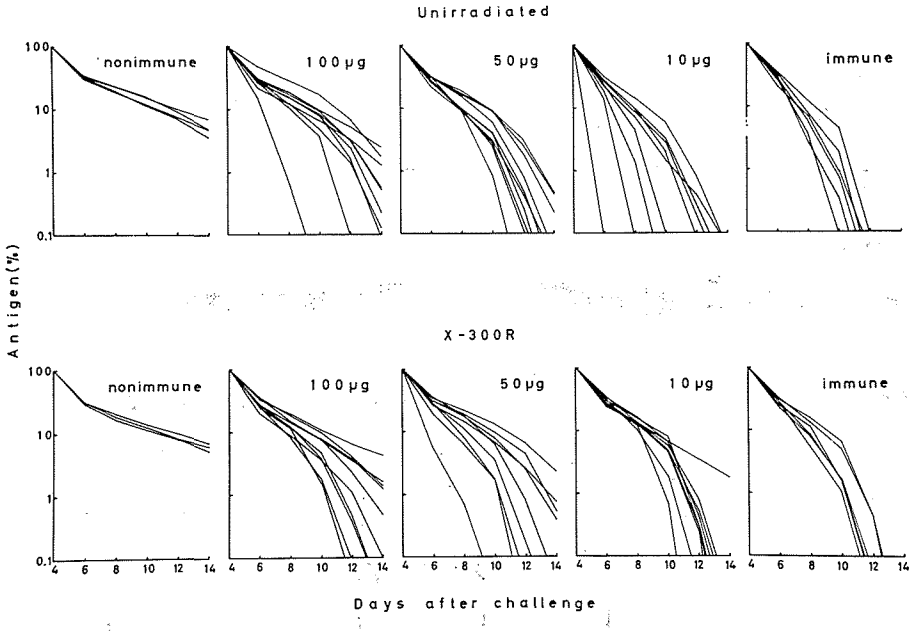

Fig. 4. Detailed and repeated examinations of a part of the experiment shown in Figure 3.

experiment of Figure 3 was repeated increasing the number of test animals in each subgroup. The 200 R-irradiation was not done in this case.

In Figure 3 and Figure 4, it was indicated that the elimination rates of I*-BGG in the nonimmune and the immune subgroups scarcely varied with individuals. The individual variation in the elimination rate was seen in the subgroups in which mice had been treated with sBGG. The effectiveness for inducing the unresponsiveness tended to increase, irrespective of the irradiation, as the dose of sBGG increased.

The question if the specific immunologic unresponsiveness caused by the treatment of sBGG was induced more readily in irradiated mice than in unirradiated mice cannot be immediately answered from the graphs in these figures. Somewhat lower rates of the elimination, if anything, might be recognized in the irradiated groups, for example, the mice in subgroups administered with 10 μg of sBGG in Figure 4. However, the direct comparison of the elimination patterns between the irradiated and the unirradiated groups seems impossible, since also the elimination rates in the immune subgroups of irradiated mice were somewhat lower than unirradiated mice. For answering the question, some correction for compensating the x-ray effect on the normal immune response must be necessary. For this purpose, the negativity index was tentatively devised to give a normalized expression. The method of calculation for converting the elimination rate into the index value is shown in Figure 5.

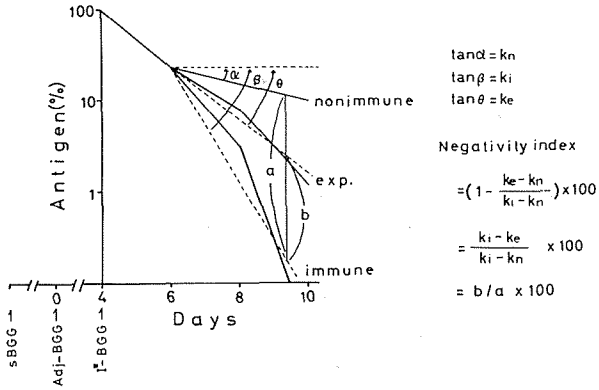

Fig. 5. The method of calculation for converting the rate of antigen elimination from the absolute value to the relative value expressed in terms of negativity index.

From the experimental results in Figure 3 and Figure 4, the antigen elimination rates of mice treated with sBGG were converted into negativity indices. The mean elimination rates of the nonimmune and those of the immune subgroups served as references. The negativity indices converted from the antigen

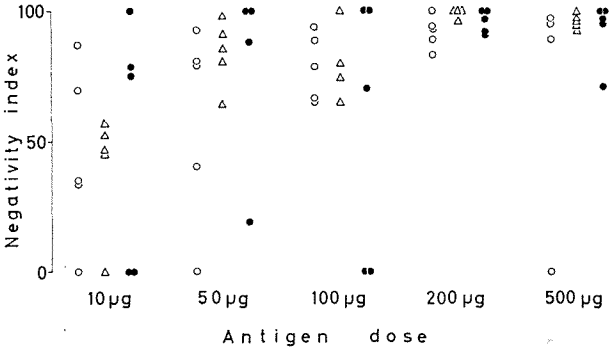

Fig. 6 Negativity indices calculated from the elimination rates of mice injected with sBGG. Each point corresponds to one line in Figure 3. o : Unirradiated, △ : x-200 R, ● : x-300 R.

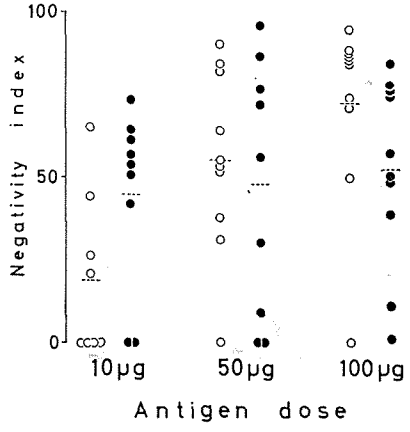

Fig. 7. Negativity indices calculated from the elimination rates of mice injected with sBGG. Each point corresponds to one line in Figure 4. o : Unirradiated, ● : x-300 R. Horizontal bars of broken lines represent mean values.

elimination rates in Figure 3 and Figure 4 are dotted in Figure 6 and Figure 7 respectively. It seems difficult, only at a glance, to find any appreciable difference in the level of the values between irradiated and unirradiated mice in each subgroup. The distribution and the mean of index values in Figure 7 were examined statistically. In any subgroup, no significant difference at the 5% level was found both in the distribution and in the mean value : F values were 1.20 ($P > 0.2$), 1.70 ($P > 0.2$) and 1.02 ($P > 0.2$) for the dlstribution, and 4.03 ($0.2 > P > 0.05$), 0.23 ($P > 0.2$) and 2.51 ($0.2 > P > 0.05$) for the mean values in 10 μg, 50 μg and 100 μg of sBGG-administered subgroups respectively.

The interference of Adj-BGG with the effect of sBGG. Mice were injected intraperitoneally with 1,000 μg of sBGG as a treatment for inducing the specific unresponsiveness. At the same time, immunogenic Adj-BGG containing varying acmounts of FI-BGG was administered subcutaneously. Oen day prior to injection, one third of the mice were irradiated with 300 R x-ray to examine if the irradiation might interfere with the competitive effect, if any, between sBGG and Adj-BGG. The mice injected no Adj-BGG and those injected with

Table 2

The interference of Adj-BGG with the establishment of the specific immunologic unresponsiveness which may be induced by 1,000 μg of sBGG

X-Ray (R)	Amounts of FI-BGG (μg) in Adj-BGG[a]	Negativity Index			
		> 67	67 - 33	33 - - 50	-50>
0	Not Injected	8/10[b]	1/10	1/10	-
	1	3/5	-	2/5	-
	10	5/10	1/10	-	4/10
	100	4/1;	-	-	6/10
	1000	-	-	-	9/9
300[c]	Not Injected	5/5	-	-	-
	1	3/5	-	1/5	1/5
	10	3/6	-	1/6	2/6
	100	1/6	2/6	2/6	1/6
	1000	-	-	-	5/5
0	0[d]	5/5	-	-	-
	1000[e]	4/5	1/5	-	-

a Varying amounts of FI-BGG were incorporated into 0.3 ml of the adjuvant emulsion, and they were injected subcutaneously at the time of intraperitoneal injection of 1.000 μg of sBGG.

b Number of mice per total number in each experimental group.

c Given 24 hours before the injectionof sBGG.

d Injected with 0.3 ml of the adjuvant emulsion containing no FI-BGG.

e Given 6 days after the injection of sBGG.

adjuvant emulsion including no FI-BGG served as controls. One group of mice was injected with Adj-BGG 6 days after the injection of sBGG. Challenge injection and the I*-BGG administration were performed according to the standard schedule. The results are shown in Table 2. The values of the negativity index were assorted into 4 classes : complete or nearly complete unresponsiveness (>67) ; partial unresponsiveness (67-33) ; occurrence of either the primary response or the low rate secondary response elicited by the challenge injection (33--50); the secondary response attributable to the primary Adj-BGG injection and the challenge injection (<-50). The indications from Table II were as follows:a) the immunogenic form of the antigen, Adj-BGG, interfered with the induction of the specific unresponsiveness due to 1,000 μg of sBGG; b) 300 R x-ray hardly affected both the induction of the specific unresponsiveness and the antagonistic action of Adj-BGG against sBGG ; c) the degree of the interference of Adj-BGG with the immunosuppressive action of sBGG tended to increase with the dose of the antigen in Adj-BGG ; d) such an interferring effect of Adj-BGG was actually ascribed to the antigen itself in immunogenic form, since the adjuvant emulsion containing no FI-BGG could not interrupt the action of sBGG; e) the state of the unresponsiveness seemed to be established by 6 days after the injection of sBGG, since the administration of Adj-BGG at that time could not reverse the unresponsive state.

Discussion

The following two major factors are possible to be involved in the establishment of the specific immunologic unresponsiveness : a) the degree of the immunologic competence or potentiality of animals, that is, the population of the immunologically competent cells which is considerably small in younger age and is reduced by x-irradiation or radiomimetics in adult animals, b) the concentration of antigen in extracellular space which may be determined not only by the dose of administration into animals but the rate of the nonspecific and specific elimination from the circulation.

The present investigation aims at comparing the amount of antigen required for suppressing the antibody response specifically between the normal adult animals and the immunologically less competent animals caused by x-irradiation. The attention was paid especially at the factor of b) mentioned above. The employment of adult mice and that of nonimmunogenic sBGG as suppressive agent was anticipated to ensure approximately the equal concentration of antigen both in normal and irradiated mice, since the body size and the rate of elimination of sBGG were not affected significantly by x-irradiation.

The present experiment indicated that the establishment of the immunologic unresponsiveness was dependent on the concentration of antigen in the extracellular space which increased as the dose of antigen given to animals increased. The concentration of antigen may determine, with some equilibrium factors, the

amount of antigen attacking each of the competent cells. The number of the immunologically competent cells does not seem to have any direct correlation with the relative difficulty in the induction of the immunologic unresponsiveness. This was realized from Figure 6 and Figure 7 in which the comparison of the negativity indices between normal and irradiated mice in each subgroup did not represent any statistically significant difference.

The argument mentioned above does not necessarily exclude the possibility that the immunologically less competent animals are prone to be suppressed by the antigen in amounts relatively smaller than that required for the normal animals. The majority of cases so far reported (2-4, 10-12, 16, 17) have employed the immunogenic form as test antigens. In general, the immunogenic antigens may be ready to be processed by phagocytic cells and also may stimulate the antibody response to be cleared efficiently in normal adult animals, while in immunologically less competent animals the processing of antigens may also be less effective (14, 15) so that the antigen may last during considerably longer period to suppress the antibody response. When adult animals which were irradiated or administered with inhibitory drugs were employed as the immunologically inadequate animals, the discrimination must be done between the nonspecific suppressive effect of these agents on antibody response and the low degree response ascribed to the specific suppressive effect of antigen. It seems that this has not been attempted in much of information so far reported. Hence, the fact that immunologically inadequate animals are prone to be suppressed by relatively small amounts of antigens seems to be only superficial, although the application of the phenomenon to the medical practice should be very fruitful.

The results of the present investigation are not inconsistent essentially with those of some other relevant experiments (18, 21). Siskind and Howard (18) reported that the threshold amount of pneumococcal polysaccharide in the induction of immunologic unresponsiveness in adult mice was unaltered by preimmunization. Weigle (21) could not found any difference between unirradiated and x-irradiated neonatal rabbits with respect to ease of induction of the unresponsiveness to bovine serum albumin.

That the relative difficulty to induce the immunologic unresponsiveness may be dependent upon the immunogenicity of test antigen has been realized from Table II. The results indicated that the effect of nonimmunogenic form of FI-BGG, or sBGG, was antagonistically influenced by immunogenic Adj-BGG in which despite FI-BGG was included. This suggests that there exists some qualitative differncee in the physical form of FI-BGG between sBGG and Adj-BGG. In our opinion, the latter may be the conditioned form to be processed by some cell systems, probably reticuloendotherial systems. The processed antigen, or perhaps its copy, may act as immunogenic agent to evoke the proliferation and differentiation of immunologically competent cells to form the antibody producing cells. Nonimmunogenic form of antigen, however, seems difficult to undergo such processing and may act directly as a immunosuppressive agent to the competent cells (cf. 20). The competition between such two

types of antigenic information may occur when immunogenic and nonimmunogenic forms are administered simultaneously. The competition appeared also in 300 R-irradiated mice, so that the large doses of x-ray might be preferable to suppress profoundly the immunogen-processing ability. This seemed practically improper for the present purpose, since the large dose of x-ray would also suppress strongly the antibody response against the challenge immunization. After the unresponsive state has been established, even the immunogenic antigen cannot reverse the situation (see the bottom column in Table 2). This may explain the memory or persistence of unresponsiveness during some period.

The above model applies well the requirement of very large amount of immunogenic antigen for paralyzing the normal adult animals (5, 9). In this case, the competition may occur between the immunogenic information emanated from the antigen-processing systems and the raw unprocessed antigen. Although the immunogenic antigens may be innately prone to be treated by the antigen-processing systems to elicit the antibody response, the large amount of antigen beyond the processing capacity is assumed to cause the direct attack of the raw antigen to the immunologically competent cells. In newborns and irradiated animals, antigen-processing systems may be less powerful, hence, even the relatively small amounts of raw antigens would be satisfactory to overcome the immunogenic information.

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