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IMMUNOLOGICAL UNRESPONSIVENESS IN MICE. I. IMMUNOLOGICAL UNRESPONSIVENESS INDUCED IN EMBRYONIC MICE BY MATERNOFETAL TRANSFER OF HUMAN γ-GLOBULIN

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SUMMARY Immunological unresponsiveness to human γ-globulin (HγG) was induced in fetal mice by maternofetal transfer after a single injection of tolerogen (deaggregated HγG) into the mothers. The degree and duration of this unresponsiveness depended upon the dose of tolerogen injected and the time of its administration during pregnancy.

Injection of 5 mg of tolerogen into pregnant mice on the 14–15th day of gestation or later caused stable unresponsiveness in their offspring which lasted for more than 56 weeks after challenge with HγG in Freund's incomplete adjuvant. However, injection of tolerogen before the 14–15th day of gestation, resulted in transient unresponsiveness in the offspring, followed by antibody formation. This unresponsiveness was specific to the tolerogen and was not terminated by challenge with a cross-reacting antigen, such as rabbit γ-globulin. When the challenge was given in Freund's complete adjuvant, only injection on the 14th day resulted in lasting unresponsiveness. Thus the 14–15th day seems to be a critical stage for induction of the immunological unresponsiveness. The significance of these results is discussed in the relation of the ontogenic development of immunocompetent cells, that is, thymus-derived cells and bone marrow-derived cells.

Similar results were obtained in rats, but no relationship could be found between the time of induction of unresponsiveness and its specificity.

INTRODUCTION

There are several reports of attempts to induce immunological unresponsiveness (or tolerance) of humoral antibody responses by prenatal exposure alone to soluble protein antigens (Dresser and Mitchison, 1968). Induction of unresponsiveness has mainly been demonstrated in chickens (Hirata and Schechtman,

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1960; Tempelis, Wolfe and Mueller, 1958) and not seen in mammals (Hanan and Oyama, 1954; Downe, 1955), though there have been a few experiments on rabbits (Trench, Gardner and Green, 1964). So far no studies on unresponsiveness in embryo mice have been reported.

As is well known, it is relatively simple to produce unresponsiveness by neonatal injection of a variety of soluble antigens into mice and it is generally accepted that such susceptibility to tolerance may be due to immaturity of the immune system in neonatal animals (Burnet, 1969). Therefore, more younger embryos could be tolerated more readily on contact with a certain amount of antigen.

In mice, the cellular events involved in the immune response have been well characterized. For antibody response to antigens, in general, the cooperation of at least two specific cell types is required. (Mitchell and Miller, 1968; Claman and Chaperon, 1969; Rajewsky et al., 1969). One type is bone marrow-derived lymphocytes (B cells) which are the direct precursors of antibody-forming cells and respond to the antigenic determinant on a given antigen. The other type is thymus-derived lymphocytes (T cells) which respond to the carrier portion of the antigen and have helper function for B cells (Mitchell and Miller, 1968). Moreover, the immunological unresponsiveness induced by an antigen (i.e. tolerogen) is understood to be a function of the unresponsiveness of T or B cells (Miller and Mitchell, 1969; Taylor, 1969; Playfair, 1969; Roelants and Goodman, 1970; Chiller, Habicht and Weigle, 1970).

Recently, much attention has been focused on the ontogenic development of the immune system in embryonic mice (Decker et al., 1972; Dwyer and Mackay, 1972; Chiscon and Golub, 1972; Nossal and Pike, 1972, 1973; Spear et al., 1973). The times of appearance and the kinetic pattern of T cells, B cells and specific antigen-binding cells in various organs have been studied by measuring the reactivities of test cells to antibodies specific to T or B cells and radioactive antigens at different embryonic ages. The development of the functions of T or B cells have also been investigated by determination of the ability of test cells to cooperate with adult B or T cells. However, there are no reports on the onset of susceptibility to tolerance of T or B cells in embryonic mice.

In studies on this problem we plan to induce unresponsiveness to a given antigen as early during embryonic life as possible and to see when susceptiblity to tolerance starts and whether the time is the same for different antigens. For this purpose, $H_{\gamma}G$ seemed a suitable antigen because this protein can be transferred across the hemochorial barrier (Gitlin and Koch, 1968; Gitlin and Morphis, 1969; Morphis and Gitlin, 1970) and because $H_{\gamma}G$ in its monomeric form (deaggregated $H_{\gamma}G$) has been widely used as a tolerogen in experiments on immunological tolerance (Dresser 1962; Golub and Weigle, 1967a, 1967b; Chiller et al., 1970).

This paper reports that unresponsiveness to $H_{\gamma}G$ could be induced in embryonic mice by its maternofetal transfer between the 14th day of gestation and term. This unresponsiveness was lost on challenge with Freund's complete adjuvant. From these results it is suggested that the cellular development of unresponsiveness varies with the time of its induction.

MATERIALS AND METHODS

1. *Animals*

Mice of the C3H strain were obtained from the Shionogi Lab., Osaka, and the Donryu rats from Nihon Rat Co., Saitama. They were fed on stock diet MF obtained from the Oriental Kobo Co., Osaka. Breeding animals were kept as groups of 20 female and 5 male mice per cage and 5 female and 2 male rats per cage and females were examined daily for vaginal plugs. Females possessing the plug were removed, caged separately, and treated with the tolerogen at the required time of gestation. The offspring were kept with their mothers until 4 weeks of age and then separated. The offspring were not segregated according to sex.

2. *Reagents*

Cohn fraction II of human γ-globulin (HγG) and rabbit γ-globulin (RγG) were obtained from AB KABI, Stockholm, Sweden. Both γ-globulins were purified by two chromatographies on a column of DEAE-cellulose. Purified γ-globulins (I*g*G) from monkey (Mon), horse (Hor), bovine (Bov), sheep (Sh) and goat (Goa), were obtained by same procedure from their globulin fractions. These sera were obtained from Kanonji Institute, The Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa. Six times recrystallized hen egg-white lysozyme (HL) were purchased from the Seikagakukogyo Co. Ltd., Osaka.

Two fragments, Fab and Fc, of HγG were prepared by digestion of HγG with crystalline mercuripapain, following the procedure of Porter (1959). These fractions were purified by the methods of Edelman (1960).

3. *Protein concentration*

Protein concentration were determined from the absorption at 280 mμ, taking the $E^{1\%}_{1cm}$ values of HγG, RγG and HL as 14, 15 and 26.4, respectively.

4. *Induction of unresponsiveness*

Pregnant 6 month-old mice or rats were given a single intraveneous injection of deaggregated protein (d-HγG or d-RγG, referred to as tolerogen) in 0.1 ml of phosphate buffered saline, pH 7.0 (PBS-7). The protein, 5% solution in PBS-7, was deaggregated by centrifugation at 100,000$\times g$ for 120 min in the SW 50 rotor of a Beckman ultracentrifuge. The upper third of the preparation was diluted with PBS-7 and used as tolerogen. In mice, the day of gestation when tolerogen was injected was estimated, taking the day when the litter was found as day 21 of gestation. On the average the period of gestation was 20 days.

5. *Immunization procedures*

The offspring of mothers which had received the tolerogen during pregnancy were immunized at 4 or 8 weeks of age, by subcutaneous injection of 50 μg (for mice) or 500 μg (for rats) of the protein in 0.1 ml of Freund's incomplete (FICA) or complete adjuvant (FCA). Booster injections were given 4 and 26 weeks after the primary immunization (referred to as challenge) using the same antigen in FICA. In

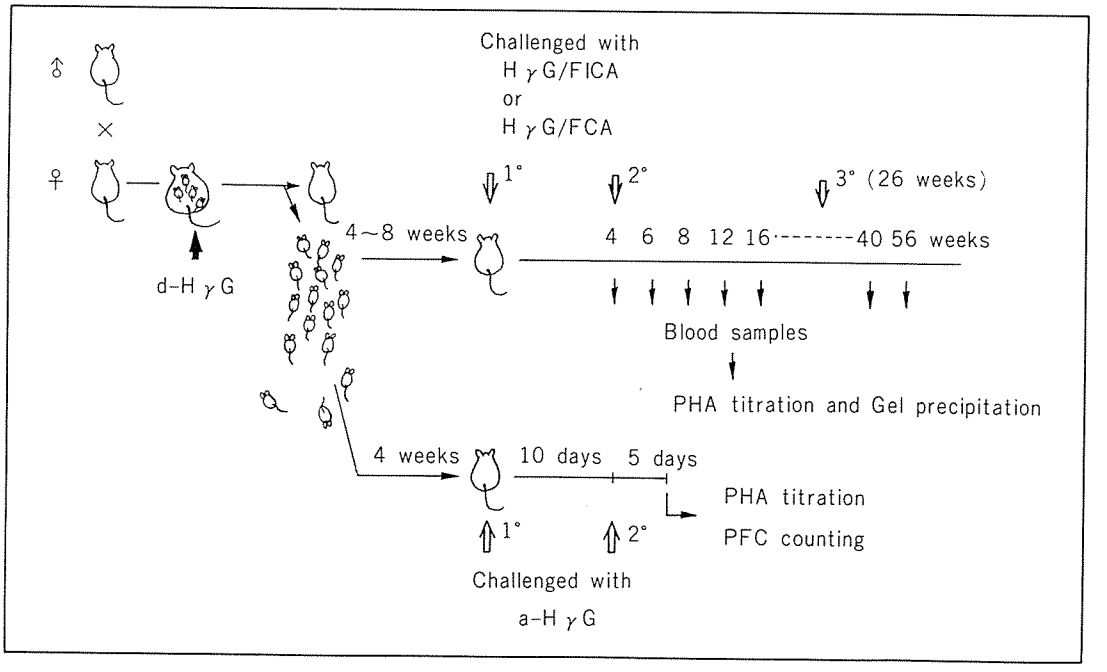

FIGURE 1. *Outline of schedule for induction of unresponsiveness, immunization and bleeding of mice or rats.*

some experiments, heat-aggregated HγG (a-HγG) was used to immunized mice. Heat-aggregation was performed by Gamble's methods (Gamble, 1966). The method of immunization used was as described by Chiller (1971).

6. *Determination of serum antibody level*

At intervals after the challenge, blood was taken from the ophthalmic vein of mice and the tail of rats. The antibody titers of the sera were determined by the passive hemagglutination (PHA) test, using 0.5% sheep erythrocytes coated with the antigen by glutaraldehyde (Segre and Segre, 1968). Results are expressed as $\log_2$ (PHA titer)$^{-1}$. When diluted serum was used, the dilution factor is shown. PHA titers of groups of mice or rats are expressed as geometric means with the standard deviations.

7. *Hemolytic plaque assay*

The method of Jerne and Nordin (1963) was used with the modifications of Cunningham and Szenberg (1968). HγG was covalently coupled to indicator sheep erythrocytes with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (Golub et al., 1968), obtained from the K & K Lab. Inc., Plainview, N. Y.. Indirect plaque-forming cells (PFC) were developed using an optimal concentration of goat anti-mouse γ-globulin.

The schedule for induction of unresponsiveness, immunization and bleeding of mice and rats is outlined in Fig. 1.

RESULTS

1. *Immunological unresponsiveness induced in fetal mice*

1) Effect of the dose of tolerogen on induction of immunological unresponsiveness in fetal mice

Three groups of mice in late pregnancy were given single intraveneous injections of 0.1 ml of solutions containing 5 mg, 0.5 mg and 0.05 mg of d-HγG, respectively and their offspring were challenged by subcutaneous injection of 50 μg of HγG or HL in FICA at 8 weeks of age. Four weeks later, sera were taken and their PHA titers against HγG were examined. As shown in Table 1, treatment of mothers in

TABLE 1. *Effect of the dose of d-HγG injected into pregnant mice on the induction of unresponsiveness to HγG in fetal mice*[a]

HγG treatment		Litter No.	Challenge antigen HγG		HL
Dose	Day of gestation		PHA[b] titer against HγG	PHA[b] titer against RγG	PHA[b] titer against HL
(mg)		1	—	—	
			—	—	
			—	—	
		2	—	—	
			—	—	
			—	—	
5	18		—	—	
			—	—	
			—	—	
		3	—	—	2
			—	—	1
			—	—	2
			—	—	3
			—	—	
	15	4	—		3
			—		2
			—		3
	16	5	—	—	
			—	—	
0.5			—	—	
		6	—	—	
	18		—	—	
			—	—	
		7	—	—	
			—	—	
			—	—	
		8	3	—	
			2	—	
0.05	18		1	—	
		9	4	1	
			5	—	
		10	3	2	3
			5	2	2
			7	3	1
			7	4	3
None		11	3	—	
			6	4	
			7	—	2
			7	—	
		12	7	1	
			7	2	

[a] Mice were challenged subcutaneously with 50 μg of HγG or HL in FICA.

[b] Values are the PHA titers of 10-fold diluted sera taken from the mice 4 weeks after the challenge. (—), PHA titer below 1 : 10.

late pregnancy with 5 or 0.5 mg of d-HγG induce complete unresponsiveness to HγG in the offspring, while 0.05 mg of d-HγG induced partial unresponsiveness.

This unresponsiveness was specific to HγG, and the immune response to HL was not suppressed.

2) Effect of time of administration of tolerogen on persistence of the state of the unresponsiveness

Six groups of pregnant mice were given a single intraveneous injections of 5 mg of d-HγG on days (9, 13, 14, 15, 18 and 20) of gestation, respectively, and their offspring were challenged by subcutaneous injection of 50 μg of HγG in FICA at 4 weeks of age and given booster injections 4 and 26 weeks after the challenge. The offspring of untreated mice were used as controls. The PHA titers to HγG in the sera of these mice were followed until 56 weeks after the challenge. Fig. 2 shows the changes in the PHA titers in each group. Four weeks after the challenge, no experimental groups showed antibody titers against HγG although the control group showed a significant antibody titer. After the booster injection, offspring of mice injected with tolerogen on the 9th day of gestation (9 day-group) began to produce antibodies and later some mice in the 13 day-group also began to show antibody titers. However, most mice in the 14, 15 and 18 day-groups remained unresponsive until at least 56 weeks after the challenge. Mice in the 20 day-group also showed prolonged unresponsiveness, but it was not so complete as in the above groups.

3) Effect of cross-reacting I*g*G on the state of unresponsiveness

Two groups of offspring from mice injected with 5 mg of d-HγG on the 16th and 18th day of gestation respectively were subdivided into two sections, and these were challenged subcutaneously with 50 μg of HγG and RγG in FICA at 4 weeks of age, respectively. A further two groups of mice from mothers injected with 5 mg of d-RγG on days 16 and 18 were treated similarly. Sera were taken 4 to 20 weeks after the challenge and antibody titers against both HγG and RγG were determined by PHA tests. The antibody titers 12 weeks after the challenge are given in Fig. 3.

The results show that the offspring of mice treated with d-HγG on day 16 or 18 day of gestation and challenged with HγG showed complete unresponsiveness to both HγG and and cross-reacting RγG. However, mice challenge with RγG demonstrated slightly suppressed responses to RγG compared with control mice but no antibody titers to HγG. On the other hand, offspring of mice treated with d-RγG on day 16 or 18 of gestation and challenged with RγG demonstrated almost no antibody titers to either antigen, whereas those challenged with HγG manifested a normal immune response to HγG but did not produce any detectable antibody to RγG. The degree of serological cross-reactivity between HγG and RγG in control groups was very low.

Thus, it did not seem possible to terminate the state of unresponsiveness by challenge with cross-reacting antigen, using any combination of antigens.

4) Effect of adjuvant on persistence of unresponsiveness

Three groups of offspring of mice which had received 5 mg of d-HγG on the 14th, 16th and 18th day of gestation were each subdivided into two sections. One section from each group was challenged with HγG in FICA and the other section with the same antigen in FCA at 4 weeks of age. The antibody titers against HγG 4 to 20 weeks after the challenge are shown in Fig. 4.

Mice challenged with HγG in FICA showed persistence of the state of complete unresponsiveness until at least 20 weeks after the challenge, whereas mice challenged with HγG in FCA showed different patterns of unresponsiveness. Mice treated at the 16th or 18th day of embryonic life started to show antibody titers 8 weeks after the challenge and these titers increased progressively with time, but mice treated at the 14th day of embryonic life did not show any antibody titer for at least

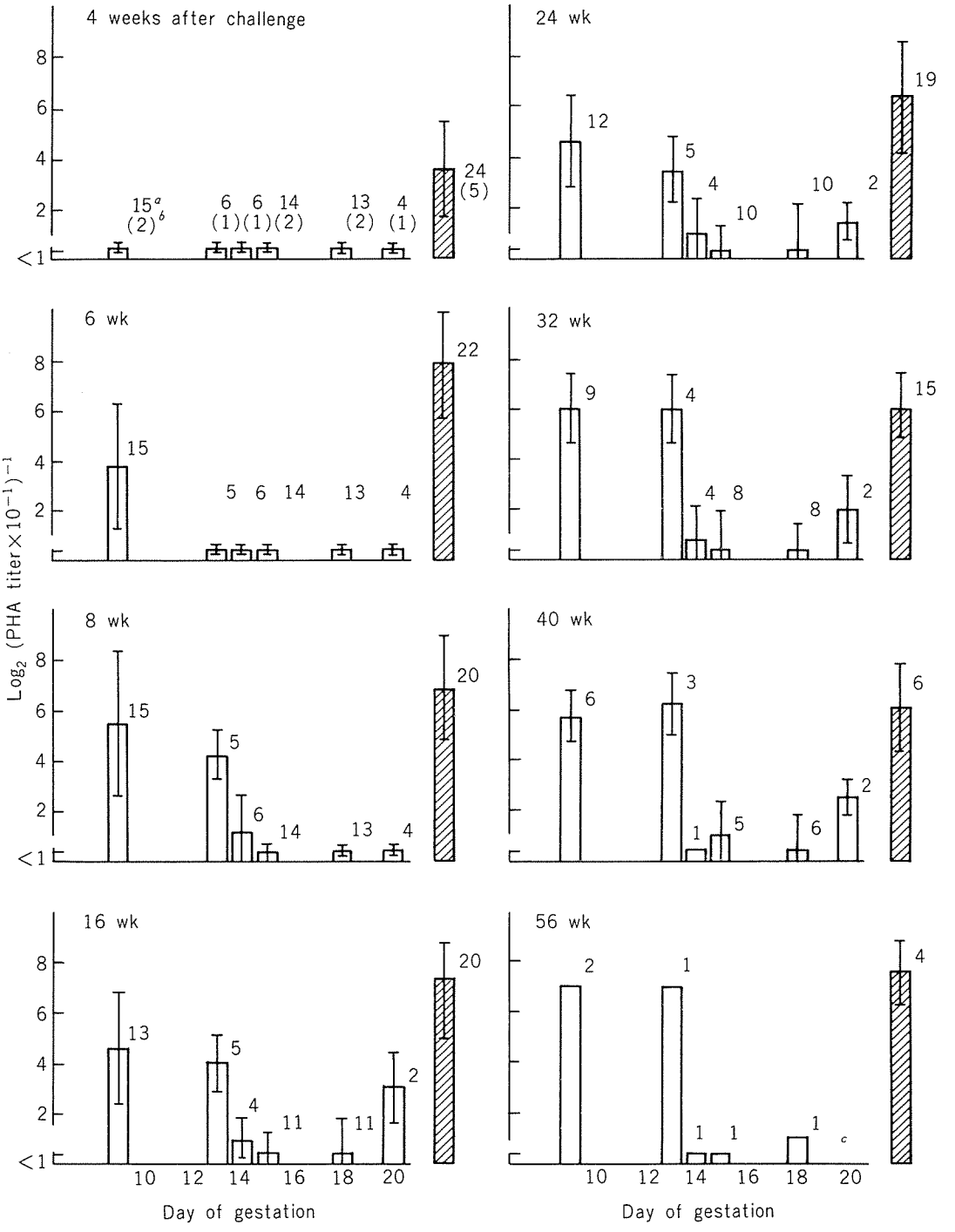
4 weeks after challenge
24 wk
6 wk
32 wk
8 wk
40 wk
16 wk
56 wk
Log2 (PHA titer×10⁻¹)⁻¹
Day of gestation
Day of gestation

FIGURE 2. *Kinetics of serum antibody titers in mice made tolerant to HγG by maternofetal transfer at various embryonic ages and challenged with HγG in FICA. Groups of mice from mothers which had received a single injection of 5 mg of d-HγG at various days of gestation, were challenged subcutaneously with 50 μg of HγG in FICA at 4 weeks of age and given booster injections 4 and 26 weeks after the challenge. Values are geometric mean PHA titers against HγG of the groups after the challenge. Standard deviations are shown as vertical bars. Shaded columns represent the mean PHA titers of control groups. a: Numbers shown above each column are numbers of mice per group. b: Numbers in parentheses are numbers of litters in each group. c: All mice in this group died 40–56 weeks after the challenge.*

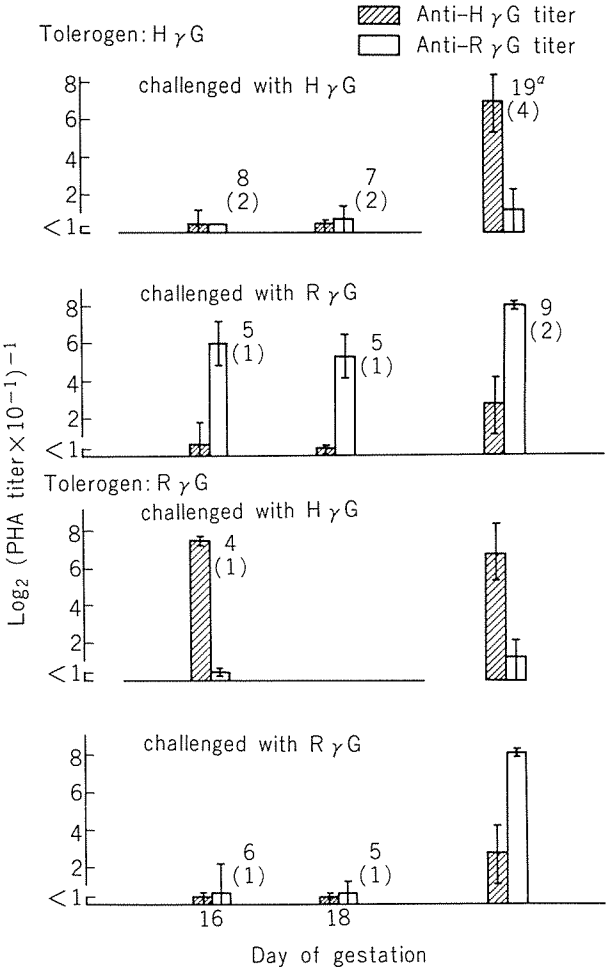

FIGURE 3. *Specificity of unresponsiveness to HγG and RγG induced in fetal mice. Groups of mice from mothers which had received a single injection of 5 mg of d-HγG or d-RγG on the 16th day or 18th day of gestation, respectively, were challenged with 50 μg of HγG or RγG in FICA at 4 weeks of age and serum antibody titers to both HγG and RγG were determined 12 weeks after the challenge. For details, see Fig. 2. a: The two columns on the right show mean PHA titers of control groups.*

20 weeks after the challenge.

5) Absence of antibody-forming cells inmice treated during the embryonic period

Next these results were compared with results on adult mice treated with d-HγG and challenged with heat-aggregated HγG (a-HγG) in saline, and studies were made to see whether the state of unresponsiveness induced in fetal mice was due to deficiency of antibody forming cells. For these purposes experiments were made using a-HγG without Freund's adjuvant as the immunogen and the state of unresponsiveness was examined by the PHA test and the enumeration of plaque forming cells. Two groups of mice treated with d-HγG on the 14th and 18th days of embryonic life, respectively, were injected intraveneouly with 400 μg of a-HγG at 4 weeks old and ten days later were given a booster injection of 400 μg of a-HγG intraperitoneally. Their sera and spleens were examined 5 days after the booster injection. As can be seen in Table 2, neither serum antibody nor 19S and 7S antibody forming cells, represented as direct and indirect PFC, respectively, were detected in the tolerant mice.

2. *Immunological unresponsiveness induced in fetal rats*

Similar results to those described above were obtained in preliminary experiments using Donryu rats. So an attempt was made to induce partial tolerance in fetal rats by maternofetal

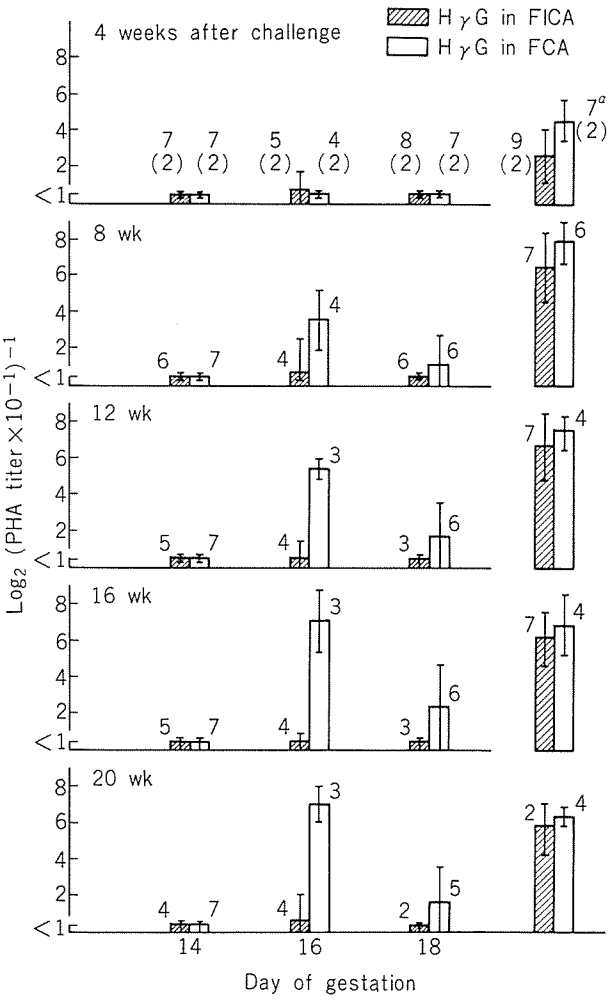

FIGURE 4. *Effect of mycobacteria in the adjuvant on persistence of unresponsiveness induced in fetal mice. Three groups of mice from mothers which had received 5 mg of d-HγG at different days of gestation were challenged with HγG in FICA or in FCA at 4 weeks of age and the serum antibody titers against HγG were determined 4 to 20 weeks after the the challenge. For details, see Fig. 2. a: The two columns on the right are mean PHA titers of control groups.*

transfer of a decreased dose of the tolerogen per body weight and to analyze the specificity of the antibody produced as a result of this partial tolerance. As the rats showed strong antibody response against HγG compared with C3H mice, it was possible to analyze the specificity of it by immunodiffusion.

Eight week old rats whose mothers had been treated with 0.5 mg or 5 mg of d-HγG at various times during pregnancy were challenged with 500 μg of HγG in FICA and sera were taken 4, 8 and 16 weeks after the challenge and analyzed by the PHA test and by immunodiffusion using Fab or Fc of HγG and various cross-reacting IgGs.

As seen in Fig. 5 and Table 3, rats treated with d-HγG in during the embryonic period showed a state of partial unresponsiveness, that is a considerable decrease of the serum antibody titer in comparison with the control group for 16 weeks after the challenge. Precipitin tests were made on individual sera using whole molecules of HγG (IgG) and its two fragments (Fab and Fc). It was found that the sera of some rats from groups treated on the 16th or 18th day of gestation did not show any precipitation line with IgG or with the two fragments. The sera of a few rats showed precipitation lines with IgG and its fragments. The sera of most rats showed a particular state of partial unresponsiveness, termed "split tolerance", namely, their sera gave precipitation lines with Fc but not with Fab. Similar analyses were carried out using various cross-reacting IgGs and it was also noted that the sera of most rats did not give precipitation lines with any cross-reacting IgGs tested except monkeys IgG. However, a few exceptional sera reacted with several kinds of IgGs while some did not react with any IgG tested. However, no relation was found between the dose of the tolerogen or the time of induction of the unresponsiveness and the specificity of the reaction.

TABLE 2. *Serum antibody titers and number of antibody-forming cells in mice rendered tolerant to Hγ G by maternofetal transfer at various embryonic ages and challenged with heat-aggregated Hγ G*[a]

Hγ G treatment		No. of mice[b]	Hγ G PFC/10^6 spleen cells[c]		PHA titer[d] against Hγ G
Dose	Day of gestation		direct PFC	indirect PFC	
(mg) 5	14	7	4±1(*N*=4)	3±2 (*N*=5)	0
	18	7	2±1(*N*=4)	4±2 (*N*=5)	0
None		8	10±4(*N*=4)	1010±281(*N*=4)	9.3±0.7

[a] Mice were challenged intraveneously with 400 μg of a-Hγ G at 4 weeks of age and 10 days later a booster injection of the same antigen was given intraperitoneally. Five days after the booster injection, serum antibody titers and the numbers of PFC were determined.

[b] Each group consisted of 2 litters.

[c] Geometric mean numbers of PFC counted in spleen cells pooled according to group ± standard deviation. N, number of slides counted.

[d] Geometric mean PHA titer of group ± standard deviation.

DISCUSSION

An immunological unresponsive state to Hγ G, as judged by failure to form humoral antibody, was induced across the maternofetal barrier in fetal mice by a single intraveneous injection of d-Hγ G into pregnant mice. This unresponsiveness is specific to the tolerogen, and its degree and duration depend upon the dose of the tolerogen and the time of its administration during pregnancy.

The dose of tolerogen affected the state of unresponsiveness, since injection of increasing amounts (0.05, 0.5 and 5 mg) of d-Hγ G into mice in late pregnancy changed the unresponsiveness from partial to complete. It is not known how these differences in the amount of d-Hγ G injected into the pregnant mother affected the concentration of d-Hγ G transferred to the fetal mice. However, Gitlin and Koch (1968) found that on increasing the maternal serum concentration of Hγ G up to a level of 2 mg/ml, there was a rapid increase in the amount transferred to the fetus on the 15th to 17th day of gestation in Swiss albino mice.

The effect of the time of administration of tolerogen on the state of the unresponsiveness was studied using a constant amount of 5 mg of d-Hγ G as tolerogen and Hγ G in FICA as immunogen. The results indicated that between the 14th day of gestation and term, and particularly the 14–15th day, is a highly susceptive period to tolerance and the unresponsiveness induced during this period is stable, lasting for at least 56 weeks after challenge. However, the unresponsiveness induced before the 14th day is transient and gradually disappears. Therefore, about the 14–15th day of gestation seems to be a critical stage for induction of the unresponsiveness. One possible explanation of this is that before the 14th day of gestation, Hγ G cannot be transferred through the maternofetal barrier efficiently, if at all. No information is available on the catabolism of deaggregated Hγ G transferred to fetal mice by the maternofetal route. However, Morphis (1970) found that the extent of maternofetal transfer of Hγ G between the 15th day of gestation and term was 100 times that on the 11th day in Swiss albino Webster mice. Moreover, the half-life of Hγ G in adult mice was found to be only about 2 days by determining the rate of elimination of ^{131}I and ^{125}I-labeled Hγ G myeloma proteins from circulation (Spiegelberg and Grey, 1968). Thus, it is quite likely that the poor induction of stable unresponsive-

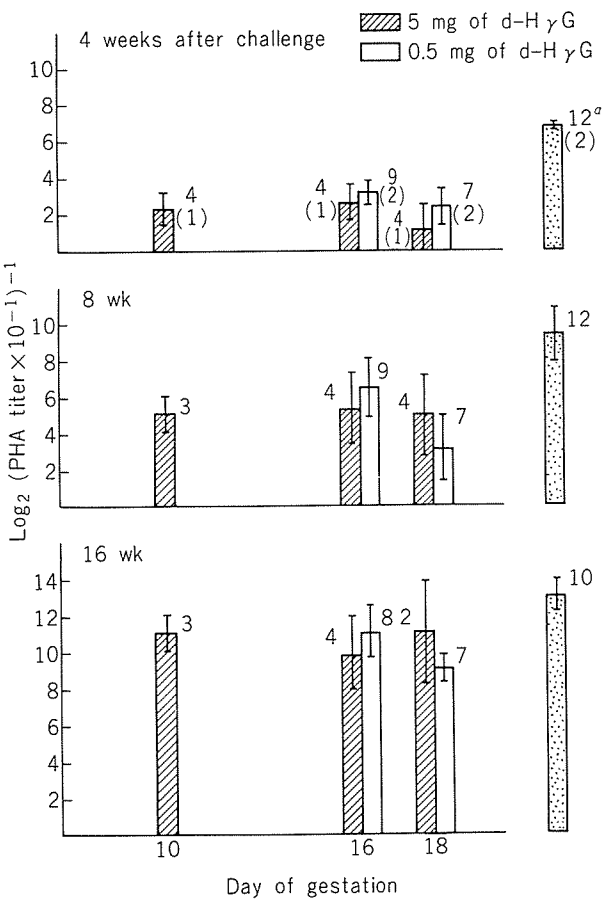

FIGURE 5. *Kinetics of serum antibody titers in rats rendered partially tolerant to HγG by maternofetal transfer at various embryonic ages and challenged with HγG in FICA. Seven groups of rats from mothers which had received a single injection of HγG at different doses at various days of gestation were challenged subcutaneously with 500 μg of HγG in FICA at 8 weeks of age and the serum antibody titers against HγG were determined 4, 8 and 16 weeks after the challenge. For details, see Fig. 2. a: The column on the right shows the mean PHA titer of the control group.*

ness before the 14th day of gestation is due to an insufficient concentration of transferred tolerogen, irrespective of whether the system of antigen recognition has develop in the embryo at this time, or not. An alternative interpretation is that the susceptibility develops from the 14th day of gestation may due to development of the immune system, involving antigenic recognition to tolerance by contact with a specific antigen (or tolerogen). Recently, there have been many reports on the ontogenic development of the immune system in embryonic mice. Dwyer and Mackay showed that specific binding of antigen to lymphocytes was first detected on thymic lymphocytes, at the 14th day of gestation in CBA mice. Spear et al. also found that T cells, B cells and antigen-binding cells appeared in the spleen of Swiss-L mice on the 15–16th day of gestation. On the other hand, Chiscon and Golub (1972) first detected B cell function capable of cooperating with adult T cells, on the 16th day of gestation in the liver of CBA mice, but could not detect T cell function in fetal or neonate liver and lung. Although there are small discrepancies between these results, in general they indicate that the immune system in fetal mice begins to develop on the 14–16th day of gestation. This is compatible with the second interpretation for the reason for a critical stage at the 14–15th day of gestation, given above.

Mice which became tolerant to HγG by maternofetal transfer of the tolerogen remained fully responsive to an unrelated antigen, such as hen egg-white lysozyme. Thus, the unresponsiveness seems to be specific to the tolerogen. However, the immune response of these mice to the cross-reacting antigen, RγG, was slightly suppressed. This suppression may be attributed to the unresponsiveness of cross-reacting T cells or/and B cells which are a minority of the total cells which respond to RγG. No antibody against HγG was detected in these mice, so the unresponsiveness to HγG was not terminated by challenge with cross-reacting antigen. These results do not agree with the report of Scales and Cruse

TABLE 3. *Specificity of antibody produced in rats rendered partially tolerant to HγG by maternofetal transfer at various embryonic ages and challenged with HγG in FICA*[a]

HγG treatment			PHA titer against HγG[b]	Precipitation with various IgGs in gel[c]								
				Human								
Dose	Day of gestation	Litter No.		IgG	Fab	Fc	Rab	Mon	Hor	Bov	Sh	Goa
(mg)			11	+	•	+	•	+	•	•	•	•
	10	1	12	+	•	+	•	+	•	•	•	•
			10	+	•	+	•	+	•	•	•	•
			12	+	•	+	•	+	•	•	•	•
5	16	2	8	±	•	•	•	•	•	•	•	•
			8	•	•	•	•	•	•	•	•	•
			12	+	•	+	•	+	•	•	•	•
	18	3	9	•	•	•	•	•	•	•	•	•
			13	+	•	+	•	+	•	•	•	•
	16	4	10	+	•	+	•	+	•	•	•	•
			9	+	•	+	•	+	•	•	•	•
			11	+	+	+	•	+	•	•	•	•
			12	+	+	+	•	+	•	•	•	•
		5	12	+	•	+	±	•	•	•	•	+
			9	+	•	•	•	•	•	•	•	•
			11	+	•	+	•	•	•	•	+	+
0.5			12	+	•	+	•	•	•	•	•	+
	18	6	8	•	•	•	•	•	•	•	•	•
			11	+	•	+	•	•	•	•	•	•
			11	•	•	•	•	•	•	•	•	•
		7	8	•	•	•	•	•	•	•	•	•
			8	+	•	+	•	+	•	•	•	•
			10	+	•	+	•	+	•	•	•	•
			6	+	•	•	•	•	•	•	•	•
None		8	13	+	+	+	+	+	+	+	+	+
			13	+	+	+	+	+	+	+	•	+
			13	+	+	+	+	+	•	•	•	•
			13	+	+	+	+	+	+	+	+	+
			14	+	+	+	+	+	+	+	+	+
			14	+	+	+	+	+	+	+	•	+
		9	12	+	•	+	+	+	+	+	+	+
			13	+	+	+	•	+	•	•	•	•
			13	+	+	+	•	+	•	+	•	+
			14	+	+	+	+	+	•	+	•	+

[a] Sera of rats of the 7 groups described in Fig. 5 taken 16 weeks after the challenge were analyzed by the PHA test and by gel precipitation.

[b] PHA titers of 10-fold diluted sera from each rat.

[c] +, strong precipitation line, ±, faint line, •, no line.

(1970) that the unresponsiveness to HγG in adult mice may be terminated by injection of bovine γ-globulin or its fragments, but the discrepancy may be due to differences in many factors, such as the phylogenic relationship among antigens, the manner of induction of unresponsiveness and the strain of mice used. Lack of termination of the unresponsiveness was probably due to unresponsiveness of B cells which respond to HγG or of cross-reacting T cells, capable of cooperating with the B cells. Further work is required on this.

Gitlin and Morphis (1969) found that there is no difference in the facilities for maternofetal transfer of HγG and RγG in mice. Further studies showed that mice which were tolerant to RγG showed almost complete unresponsiveness to homologous RγG but remained normally responsive to cross-reacting HγG. This may be because the population of cross-reacting T or B cells is only a small proportion of the total T or B cells which respond to HγG. Moreover, the unresponsiveness was not terminated by challenge with HγG. This failure may also be explained as described above.

When Freund's complete adjuvant was used for the challenge, mice which had become tolerant to HγG on the 16–18th day of embryonic life developed an antibody response 8 weeks after the challenge, but mice which had become tolerant on the 14th day did not, and the unresponsiveness persisted for at least 20 weeks after the challenge. These results can be explained on the basis of the following two predictions; first, the unresponsiveness induced on the 16 or 18th day of embryonic life may be restricted to specific T cells, while that induced on the 14th day may involve both T cells and B cells. Second, mycobacteria (H_{37}RA strain) in FCA may have a helper function for T cells, as suggested by Lind (1968) to explain his finding that unresponsiveness to a salmonella flagellin in rats can be terminated by challenge with flagellin in FCA. Recently, Chiller et al. (1973) found that bacterial endotoxin had a similar capacity to terminate the unresponsiveness of mice to HγG when administered with challenging antigen.

Previously Chiller et al. (1971) found a difference between the kinetic patterns of unresponsiveness of T cells and B cells induced in adult mice: T cells became tolerant more easily and remained for longer than B cells. A similar difference seems to exist between the susceptibilities of T cells and B cells to tolerance in fetal mice; namely, the T cells can probably easily be rendered tolerant at any time from the 14 days of gestation to term, whereas B cells are less susceptive to tolerance and in order to become tolerant a suitable amount of the tolerogen must be administered during a short critical period. The fate of B cells may be decided by various factors, such as the quantitative relationship between the concentration of tolerogen and the number of B cells or the degree of differentiation of B cells when they are exposed to the tolerogen. These prediction require further studies, including studies on the reconstitution of T and B cells. The reason for the differences found between the responses of mice which became tolerant on the 16 and the 18th days of gestation is unknown.

Heat-aggregated HγG is highly immunogenic, without added adjuvant, and has been widely employed as challenge immunogen in previous studies on unresponsiveness to HγG induced in adult mice (Weigle, 1972). In the present work this form of antigen gave similar results to those obtained using Freund's adjuvant. Complete unresponsiveness was observed in mice for at least 8 weeks after birth at both the serum and cellular level. No antibody was detected in the serum or antibody-forming cells in the spleen. This shows that the unresponsiveness observed in this work is due to lack of antibody formation, that is, absence of antibody forming cells.

Studies on the induction of the unresponsiveness by maternofetal transfer of d-HγG were also made in Donryu rats. Attempts were made to produce partial tolerance in fetal rats. This was done by inoculation of decreasing amounts of d-HγG to one tenth of

the amount employed in mice. In these partially tolerant rats, the relationship between the specificity of the depleted antibody among the anti-HγG antibodies produced and the dose of the tolerogen or time of induction of unresponsiveness was studied.

All the rats treated on the 10th day of gestation and half the rats treated on the 16th or 18th day showed the phenomenon termed "split tolerance" (Taussig, 1971). That is, gel precipitation showed that they produced detectable antibody to Fc but not to Fab. However, the analyses were not very sensitive or quantitative, so it is still uncertain whether "split tolerance" in the strict sense was actually induced. No relationship could be found using cross-reacting I*g*Gs between the specificity of the depleted antibody at the determinant level and the dose of time or inoculation of the tolerogen.

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