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A comparative study on the humoral immune responses in germ-free and conventional mice

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Summary. The plaque-forming cell (PFC) responses to sheep red blood cell (SRBC), dinitrophenyl-lysine-Ficoll (DNP-lys-Ficoll), and dinitrophenylated bovine serum albumin (DNP-BSA) have been studied in both germ-free and conventionally reared ICR mice.

In germ-free mice, the IgG response to SRBC and the IgM and IgG responses to DNP-BSA were lower than in conventional mice, but no difference was observed in the IgM response to SRBC or the IgM and IgG responses to DNP-lys-Ficoll. Further, the number of θ-bearing cells in the spleen was smaller, and the mitogenic response of spleen cells to PHA was lower in germ-free mice than in conventional mice. These observations suggest that T cells of germ-free mice remain functionally immature.

INTRODUCTION

The responsiveness of germ-free mice to various antigens has been reported to be similar to that of conventional mice (Bosma, Makinodan & Walburg, 1967; Shearer, Cudkowicz & Warburg, 1969), based on observations of direct plaque-forming cells (PFC). However, there have been contradictory results on the response to sheep red blood cells (SRBC) in terms of indirect PFC of germ-free mice (Harris, Pelc & Blackmore, 1973; Seibert, Pollard & Nordin 1974). Recently, splenocytes of germ-free mice were reported to synthesize only IgM, and it was suggested that germ-free mice lacked a population of T cells capable of inducing the 'switch' from IgM to IgG synthesis and also of suppressing IgM synthesis (Vitetta, Grundke-Iqbal, Holmes & Uhr, 1974).

In the present study, the humoral immune response of germ-free mice was more closely examined in comparison with conventional mice, using two types of antigens, thymus-independent and thymus-dependent. Also, the number of immunoglobulin (Ig)- and θ-antigen-bearing cells in the spleen, and *in vitro* responsiveness of spleen cells to T cell-specific mitogens, concanavallin A (Con A) and phytohaemagglutinin (PHA) were examined.

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MATERIALS AND METHODS

Animals

ICR germ-free and conventionally reared mice of various ages were used. They were purchased from the Central Institute for Experimental Animals, Tokyo, Japan, and bred in our laboratories. The germ-free mice were fed a sterilized solid diet (NMF solid diet, Oriental Yeast Company Ltd., Tokyo, Japan) and water. Sterility of the mice was verified each week throughout the experiment. Conventional mice were kept in open air and fed the same sterilized diet.

Antibody-forming cells in the spleen

Plaque-forming cells (PFC) were enumerated using the technique of Cunningham & Szenberg (1968). To detect anti-DNP-PFC, SRBC were coated with dinitrophenylated Fab fragment of rabbit anti-SRBC IgG (DNP_6-Fab), as described by Strausbauch, Sulica & Givol (1970). Indirect PFC (IgG-PFC) were developed by rabbit antisera to mouse IgG.

Preparation of conjugates

Ficoll (average mol. wt 400,000 daltons, Pharmacia Fine Chemicals) was dinitrophenylated by the method of Sharon, McMaster, Kask, Owens & Paul (1975), with minor modifications. In brief, Ficoll was reacted with trichloro-s-triazine (cyanuric chloride, Tokyo Chemical Industry, Tokyo, Japan) under neutral pH at 0° for 1 h, and then reacted with ε-DNP-lysine under alkaline pH at room temperature for 1 h. After extensive dialysis against water and saline, DNP-lys-Ficoll was lyophilized and weighed. Bovine serum albumin (BSA) from Armour Pharmaceuticals, Kankakee, Illinois was dinitrophenylated by the method of Eisen, Belman & Carsten (1953). The concentration of DNP groups was measured spectrophotometrically. The preparations used in the experiments reported here contained 66 moles of DNP-lysine and 7 moles of DNP per 400,000 mol. wt of Ficoll and 68,000 of BSA, respectively. For the purpose of immunization, they were administered in combination with 10^9 killed *Bordetella pertussis*.

Lymphocyte cultures

Cell suspensions were prepared for lymphocyte stimulation assay from spleens of 1–13-week old ICR germ-free and conventional mice. Spleen was gently dispersed through a stainless steel mesh and the cells were suspended in RPMI-1640 medium (Nissui Seiyaku, Tokyo, Japan), and were washed twice in RPMI-1640 medium. The cells were cultured at 1×10^7 viable nucleated cells per ml, in triplicate, in RPMI-1640 medium supplemented with 5% calf serum. Since differenct batches of calf serum manifested different results in the mitogenic stimulation of spleen cells to Con A, the same batch was used throughout the experiments presented here. Various amounts of purified Con A (Daiichi Pure Chemicals, Tokyo, Japan), PHA-p (Difco, Labs, Detroit, Michigan, control 573353), or medium was added and cultures were incubated at 37° in a humidified atmosphere of 5% CO_2 in air. Forty hours following the initial time of incubation 1 μCi of tritiated thymidine ([^{3}H]Tdr; 10 Ci/mmol, Daiichi Pure Chemicals, Tokyo, Japan) was added. Twenty-four hours later, cells were removed, washed twice with phosphate-buffered saline, and collected by filtration on glass fibre filters and washed sequentially with 10% trichloroacetic acid and methanol. The filters were dried and counted by liquid scintillation spectroscopy.

Detection of surface Ig and θ antigen

Rabbit anti-mouse IgG (RAMIG) was prepared by the ordinary method, and rabbit anti-mouse brain associated θ (RAMBAθ) was prepared by the method of Golub (1971). They were absorbed with ICR mouse erythrocytes and liver powder before use. Freshly prepared spleen cells were incubated with RAMIG or RAMBAθ at 37° for 1 h. Cells were washed twice with balanced salt solution (BSS) containing 10 mM sodium azide, then fluorescein isothiocyanate conjugated guinea-pig anti-rabbit-immunoglobulin (fluorescein:protein ratio 0·83) were added and incubated at 37° for 1 h. The cells were washed twice with BSS and examined in NIKON fluorescence microscopy (dead cells were not scored, they showed uniform, dull, internal and external fluorescence).

RESULTS

Postnatal development of antibody response to SRBC

ICR germ-free and conventional mice were injected i.p. with 10^9 SRBC at various ages between 0 and 56 days, and the numbers of IgM (Fig. 1) and IgG (Fig. 2) PFC were determined on day 4 and on day 9 respectively. IgM PFC of both germ-free and conventional mice increased exponentially during the first 3 weeks after birth and then reached a plateau (Fig. 1). Although the response of germ-free mice was always slightly lower than that of conventional mice, this difference was not always significant. As regards IgG production (Fig. 2), no detectable IgG PFC were found in both germ-free and conventional mice within the first week after birth, but their number increased abruptly and reached a plateau during the third week. In contrast to IgM PFC, IgG PFC of germ-free mice was less than that of conventional mice by a factor of about ten (Fig. 2).

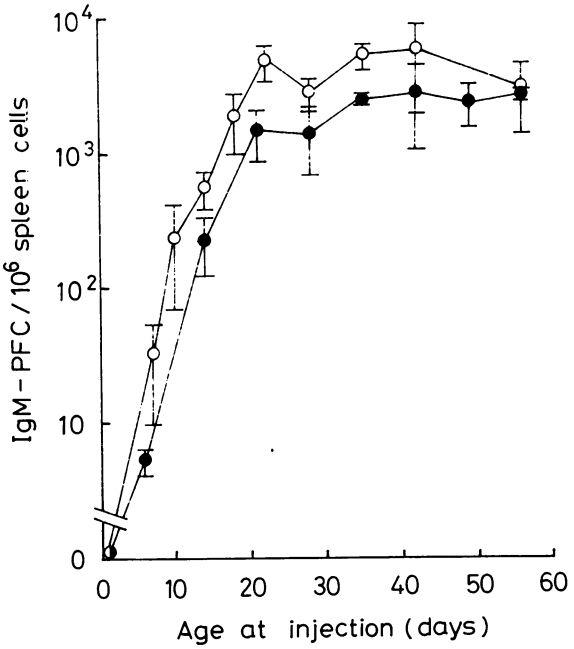

Figure 1. The anti-SRBC IgM-PFC response of germ-free (●) and conventional (○) mice of various ages. The response was measured 4 days after a single antigen injection. Each point is the arithmetic mean ± s.e. of PFC per 10^6 nucleated spleen cells.

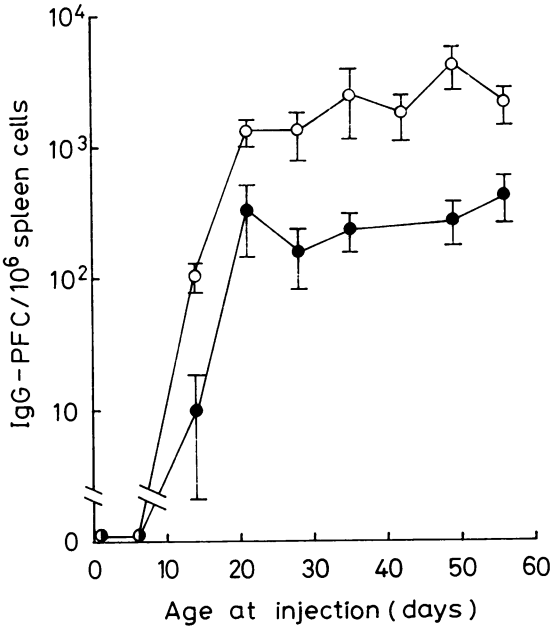

Figure 2. The anti-SRBC IgG-PFC response of germ-free (●) and conventional (○) mice of various ages. The response was measured 9 days after a single antigen injection. Each point is the arithmetic mean ± s.e. of PFC per 10^6 nucleated spleen cells.

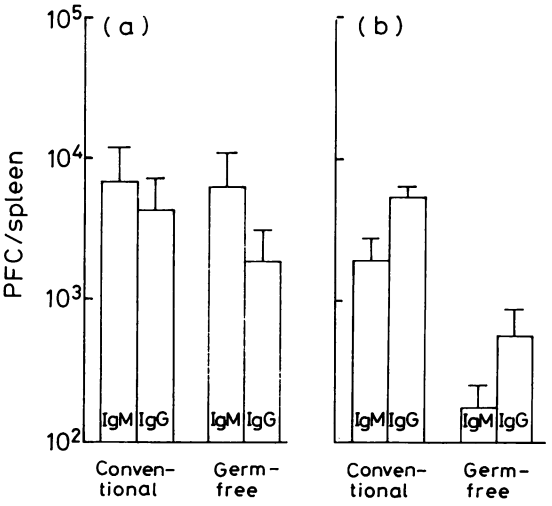

Figure 3. Primary anti-DNP response to DNP-lys-Ficoll (a) and DNP-BSA(b). Six-week-old germ-free and conventional mice were injected i.p. with 100 μg of DNP-lys-Ficoll, or 100 μg of DNP-BSA, mixed with 10^9 of *Bordetella pertussis* and the number of PFC per spleen was determined 4 days later except for the IgG response to DNP-BSA, which was determined 9 days after injection of 1 mg of DNP-BSA.

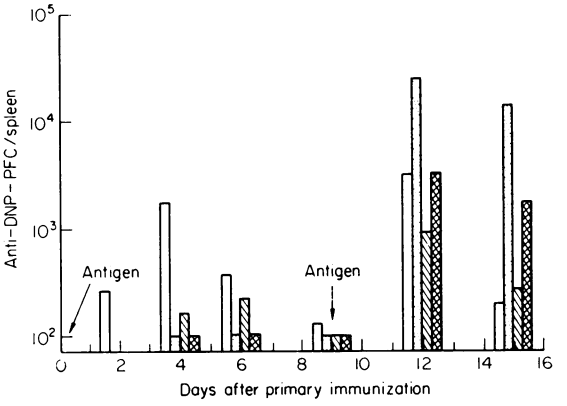

Figure 4. Secondary anti-DNP response to DNP-BSA. Six-week-old germ-free and conventional mice were injected i.p. with 100 μg of DNP-BSA, mixed with 10^9 of *Bordetella pertussis* on day 0 and again on day 9. The number of PFC per spleen were determined on days 2, 4, 6, 9, 12, and 16. Open columns (conventional IgM); stippled columns (conventional IgG); hatched columns (germ-free IgM); cross-hatched columns (germ-free IgG).

DNP-PFC response to DNP-lys-Ficoll and DNP-BSA

The responses of germ-free and conventional mice to DNP-lys-Ficoll and to DNP-BSA were examined using 6-week old mice (Fig. 3). In the case of DNP-lys-Ficoll, a thymus independent (TI) antigen (Sharon *et al.*, 1975), which was administered i.p. at 100 μg, no significant difference was found between germ-free and conventional mice in both IgM and IgG PFC (Fig. 3a). On the other hand, DNP-BSA, a thymus-dependent (TD) antigen, produced significant differences in both IgM and IgG DNP-PFC. That is, conventional mice produced about ten times more IgM and IgG-PFC than germ-free mice (Fig. 3b).

Secondary response to DNP-BSA

Six-week-old germ-free and conventional mice were injected i.p. with 100 μg of DNP-BSA on day 0 and again on day 9, and IgM and IgG DNP-PFC in the spleens were assayed after various intervals (Fig. 4). No detectable IgG response was found at this antigen dose after the primary immunization, but rapid development of IgG PFC occurred after the secondary immunization in both germ-free and conventional mice. The response in germ-free mice, however, was again significantly less than in conventional mice (Fig. 4).

Ratios of Ig-bearing and θ-bearing cells in the spleen of germ-free and conventional mice

Six-week-old normal (non-treated) germ-free and conventional mice were examined for the ratio of Ig-bearing and θ-bearing cells in the spleens by an indirect immunofluorescence technique. As seen in Table 1, a statistically significant difference was observed in the percentage of θ-bearing cells between the spleen cells of germ-free and conventional mice but not in that of Ig-bearing cells. Since the total numbers of nucleated cells in the spleen of germ-free and conventional mice were similar, germ-free mice seem to contain a smaller number of θ-bearing cells in the spleen than conventional mice.

Table 1. Percentage of immunoglobulin-bearing(Ig^+), and theta antigen-bearing(θ^+) cells in the spleen of normal germ-free and conventional mice

Mouse*	Percent of:	
	Ig^+ cells†	θ^+ cells‡
Germ-free	47·4±2·5	32·0±2·3
Conventional	42·8±2·1	40·2±3·7

* Six-week-old mice were used.

† $P = 0{\cdot}05$, ‡ $P < 0{\cdot}02$; determined by Student's t-test between germ-free and conventional group.

Response of spleen cells to T-cell mitogens

A subpopulation of Con A-reactive T cells in the spleens of adult mice, which also was PHA-reactive, has been reported (Spear & Edelman, 1973) to arise later in development. It was therefore of interest to study whether spleen cells from germ-free mice might not respond to PHA. The *in vitro* reactivity of

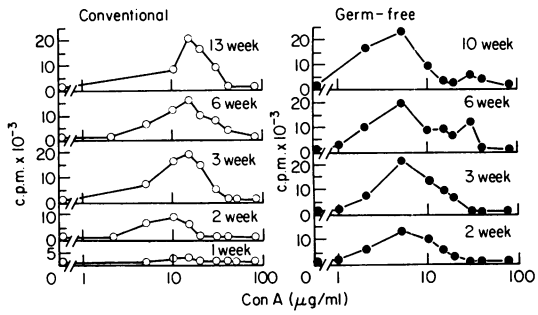

Figure 5. Mitogenic stimulation of spleen cells from germ-free (●) and conventional (○) mice of different ages as a function of Con A concentration. The results are expressed by the arithmetic mean of triplicate cultures.

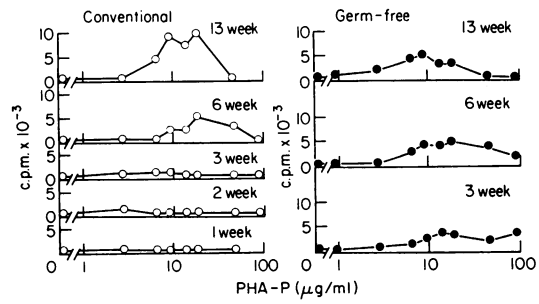

Figure 6. Mitogenic stimulation of spleen cells from germ-free (●) and conventional (○) mice of different ages as a function of PHA-p concentration. The results are expressed by the arithmetic mean of triplicate cultures.

spleen cell suspensions from germ-free and conventional mice of various ages to both two substances was therefore studied. As shown in Fig. 5, spleen cells from 2-week-old germ-free or conventional mice responded to Con A, but maximal responses were observed with cells from both germ-free and conventional mice 3 weeks old and older. The optimal dose of Con A was different in germ-free and conventional mice, being 5 and 15 μg per ml, respectively. Fig. 6 shows the response to PHA. Cells from 3-week-old or younger conventional mice did not respond, but those from older mice showed increasing responses (6 and 13 weeks). In contrast, spleen cells from germ-free mice showed only weak responses, irrespective of their age (3, 6 and 13 weeks).

DISCUSSION

The results presented above were in agreement with Harris *et al.* (1973) in that the response to SRBC in terms of indirect PFC was lower in germ-free mice than in conventional mice, whereas no difference was found in direct PFC. Other workers (Seibert *et al.*, 1974), however, observed no difference between germ-free and conventional mice either in direct or in indirect PFC response to SRBC. The reason for this discrepancy is not clear, but the age and the strain of mice used seems to be important. For instance, we observed that the serum IgG level in young germ-free mice used in the present study (up to 8 weeks) was low but increased with age, although significantly lower than in conventional mice. In studying the development of serum protein and lymphoid tissues in germ-free mice fed a chemically defined, water soluble, low mol. wt diet, Wostman, Pleasants, Bealmear & Kincade (1970) found the same gradual increase of IgG with age and concluded that antigens in the diet constitute an important source of stimulation of the lymphoid tissues.

SRBC is a complex antigen composed of TI and TD antigen, and the production of IgG is relatively more dependent on the presence of thymus-derived (T) cells than is the production of IgM (Taylor & Wortis, 1968). It may therefore be assumed that the lower IgG PFC response to SRBC in germ-free mice could be a reflection of immaturity or deficiency of T cells. To test this point more clearly, the responses of germ-free and conventional mice to TI and TD antigens were examined. The results showed, in support of the above assumption, that germ-free mice produced significantly fewer IgM and IgG PFC in both primary and secondary responses to DNP-BSA (a TD antigen) than conventional mice, whereas the IgM and IgG PFC responses to DNP-lys-Ficoll, a TI antigen (Sharon *et al.*, 1975), were similar in germ-free and conventional mice. Two additional findings may be associated with this reduced responsiveness of germ-free mice to TD antigen. Firstly, the proportion of splenic θ-bearing cells of 6-week-old germ-free mice was significantly less than in conventional mice of the same age. Secondly, only weak responses to PHA were demonstrated even in adult germ-free mice. It is thought that in mice there are two types of T cells which are reactive to Con A, one being immature T cells and the other mature ones which are also reactive to PHA and may have a helper function (Spear & Edelman, 1973; Hirst, Beverley, Kisielow, Hoffman & Oettgen, 1975). The present results may be explained by postulating that germ-free mice contain fewer helper T cells in the spleen. Although the strength of the response to Con A was similar in both germ-free and conventional mice, spleen cells from germ-free mice had an optimal concentration of Con A differing from conventional mice. Whether it represents a qualitative difference in Con A-reactive cells between germ-free and conventional mice (e.g., a difference in the number of Con A receptors on the cell surface) is currently being investigated.

The reason for this lower responsiveness to TD antigen in germ-free mice is not clear, but it is known that a stable bacterial flora is established within a few weeks after birth in the intestinal lumen of conventional mice, and it is possible that bacterial products from this flora may influence cell differentiation. In fact, bacterial lipopolysaccharides (LPS) can stimulate differentiation of T cells *in vitro* (Sheid, Hoffman, Komuro, Hämmering, Abbot, Boyse, Cohen, Hooper, Schulof & Goldstein, 1973). In our preliminary studies of the antibody response of gnotobiotic mice (e.g., *Escherichia coli* or *Lactobacillus* monocontaminated mice), we observed no differences in the IgG responses to SRBC among the conventional and the above two types of gnotobiotic mice. Further studies on the antibody response in gnotobiotic mice are now in progress in our laboratory.

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