Specific and Non-Specific Immunoglobulin Synthesis in Germfree Mice Immunized with Ferritin by Different Routes

It has repeatedly been reported that the injection of a specific antigen into an animal is followed by the appearance, in the serum, of increased amounts of immunoglobulins which apparently have no binding capacity for the antigen[1–4]. Except in the work of ASOFSKY et al.[4] which described non-specific increases of IgM in germfree mice immunized with ferritin, little information is available on the immunoglobulin classes to which such 'non-antibody' gamma globulins belong.

The present investigation was carried out with the aim of assessing, after immunization, the share in antibody and 'non-antibody' formation of 4 immunoglobulin classes (IgG_1, IgG_2, IgM, and IgA) in the mouse. Germfree mice were chosen for this purpose because of their low base-line levels of immunoglobulins.

Germfree C_3H mice, aged 3–4 months were divided into 5 groups and immunized with horse spleen ferritin (FRT) as follows: (a) 5 mice received 1×1 mg FRT i.p.; (b) 4 mice 1×1 mg FRT s.c.; (c) 4 mice 3×1 mg FRT s.c.; (d) 4 mice 3×1 mg FRT i.p., and (e) 4 mice 10 mg FRT per ml drinking water for 38 days.

The first (or only) injection in each series was administered as an emulsion in Freund's incomplete adjuvant. In those mice receiving more than 1 injection, antigen was given at 10-day intervals. Parenterally immunized mice were killed 10 days following the last (or only) injection. Orally stimulated animals were killed 18 h after removal of ferritin from the drinking water. Controls included animals having received incomplete adjuvant s.c. or i.p., as well as 1 non-injected mouse. Serum was collected by cardiac puncture and stored at 4 °C under paraffin oil, after addition of 0.1% sodium azide. This method of storage effectively prevented concentration of the samples through evaporation.

A detailed account of the method employed for the quantitation of immunoglobulins and specific antibody present in different immunoglobulin classes has been presented elsewhere[5]. Briefly, the method measures the amounts of each immunoglobulin remaining in the serum sample after specific antibody has been eliminated by the addition of excess antigen (FRT), followed by removal of this antigen by means of a heterologous (rabbit anti-FRT) antiserum. Non-precipitating as well as precipitating antibodies are measured by this technique. The quantitation of the 4 serum immunoglobulins, before and after elimination of specific antibody, was carried out by the single radial immunodiffusion method[6], using a pool of normal (conventional) C_3H mouse serum as the

[1] W. C. BOYD and H. BERNARD, J. Immun. *33*, 111 (1937).
[2] B. A. ASKONAS and J. H. HUMPHREY, Biochem. J. *68*, 252 (1958).
[3] R. A. BINAGHI, J. Immun. *97*, 159 (1966).
[4] R. ASOFSKY, N. S. IKARI and M. B. HYLTON, *Advances in Germfree Research and Gnotobiology* (Ed. M. MIYAKAWA and T. D. LUCKEY; CRC Press, Cleveland 1968), p. 219.
[5] D. R. NASH and J. F. HEREMANS, Immunology *17*, 685 (1969).
[6] G. MANCINI, A. O. CARBONARA and J. F. HEREMANS, Immunochemistry *2*, 235 (1965).

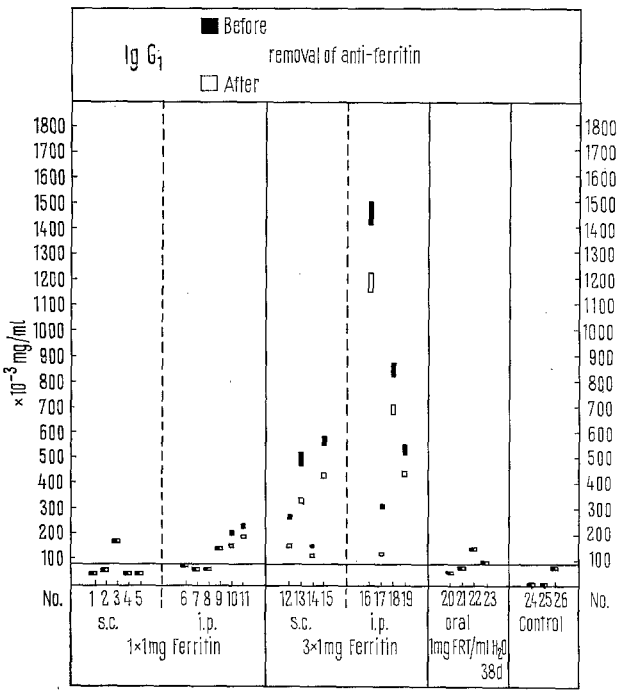

Fig. 1. Quantitative analysis of serum IgG_1 before and after removal of specific anti-ferritin antibody. The solid horizontal line represents the maximum concentration of this immunoglobulin found in the serum of non-immunized germfree C_3H mice. s.c., subcutaneous; i.p., intraperitoneal; oral, ferritin in drinking water; Control: 24, Freund's incomplete adjuvant ($1 \times$ s.c.); 25, non-injected; 26, Freund's incomplete adjuvant ($1 \times$ i.p.).

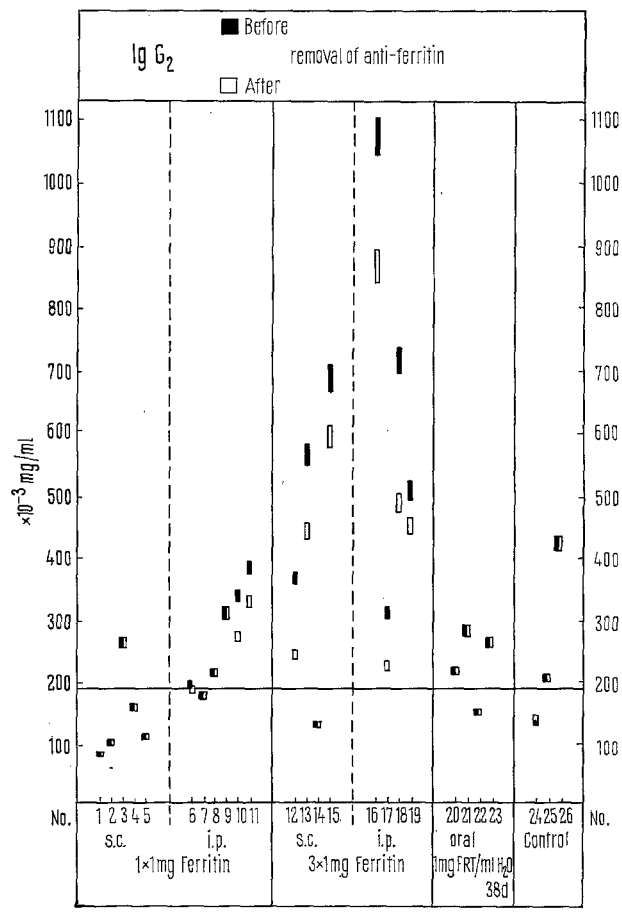

Fig. 2. Quantitative analysis of serum IgG_2 before and after removal of specific anti-ferritin antibody. See Figure 1.

standard for all Ig classes. The values given by BARTH et al.[7] for the concentrations of the different classes of immunoglobulins in normal C_3H mice were assumed to apply to this standard serum pool. The technical error of this method was found to be $\pm 3\%$ throughout a broad range of concentrations and for all 4 immunoglobulins tested.

The concentrations of IgG_1, IgG_2, IgM, and IgA in the different groups of animals and their controls, before and after elimination of specific anti-FRT antibodies, are graphically represented in Figures 1–4. Each value is indicated by a rectangle corresponding to the $\pm 3\%$ range about the actual measurement. Differences between pre- and post-absorption values (black and white rectangles, respectively) are assumed to measure antibody whenever there is no overlapping between the indicated confidence limits.

Preliminary observations (unpublished) have shown that the immunoglobulin levels in germfree C_3H mice, when determined as a percentage of the same immunoglobulin concentration in normal conventional C_3H mice, range as follows: IgG_1, 0–10%; IgG_2, 0–10%; IgM, 100 130%; IgA, 0–5%. In the test sera, immunoglobulin levels remaining after absorption (white rectangles) and exceeding the upper limits indicated above (horizontal lines in Figures 1–4), were considered to represent non-specific stimulation.

In all the 10 animals from the parenterally immunized group (19 mice) which had developed an antibody response, specific anti-ferritin activity could be allocated to the IgG_1 class of serum immunoglobulins. All the mice that had received 3 injections of FRT (Nos. 12–19) had responded by producing anti-FRT of this type (Figure 1). 'Non-anti-FRT IgG_1' was found to be increased in all animals which had developed an IgG_1 type antibody response. In addition, some of the mice (Nos. 3, 9 and 22) not responding with measurable antibody of this class had serum IgG_1 levels slightly higher than that normally found in the germfree non-immunized mouse (Figure 1).

In all but one of the animals having developed anti-FRT of the IgG_1 class (No. 14), antibodies of similar specificity could also be detected in the IgG_2 class. Again, the best responses were those obtained after repeated parenteral immunization (Figure 2). An increase in 'non-anti-FRT IgG_2' was noted in all sera having antibody of this class, as well as in 3 parenterally stimulated mice receiving 1 mg FRT (Nos. 3, 8 and 9). 3 orally stimulated animals (Nos. 20, 21 and 23) which failed to develop detectable anti-FRT IgG_2, did develop higher than expected levels of this immunoglobulin (Figure 2).

Serum antibody of the IgM class could be detected in only 4 animals (Nos. 13, 14, 17 and 19) and these had been stimulated by multiple injections of antigen (Figure 3). Large increases in 'non-anti-FRT IgM' were found in those animals that had received a single i.p. injection of antigen in incomplete adjuvant (Nos. 6–11). Smaller rises of 'non-anti-FRT IgM' were observed in several other parenterally stimulated animals, but no obvious correlation seemed to exist between the presence of specific antibody and 'non-anti-FRT IgM'. One orally stimulated mouse (No. 22) which showed a slight increase in IgG_1 but not IgG_2, had also developed a small amount of 'non-anti-FRT IgM' (Figure 3).

Antibodies of the IgA class could be demonstrated in the serum of 2 out of the 19 mice immunized by the parenteral route. Both had received their antigen i.p. (Nos. 11 and 16). In the orally stimulated group, 2 of the 4 mice had produced measurable amounts of serum IgA

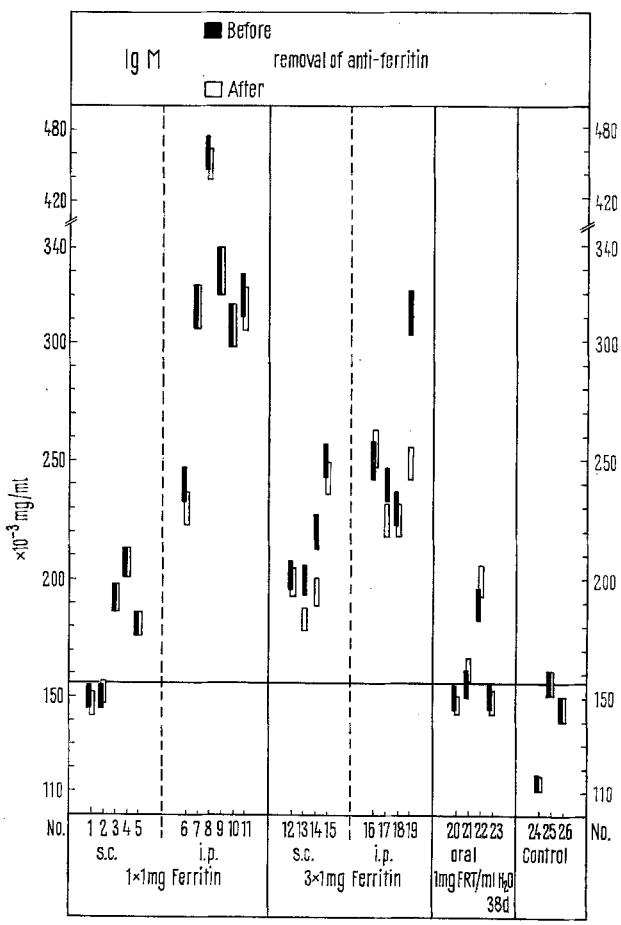

Fig. 3. Quantitative analysis of serum IgM before and after removal of specific anti-ferritin antibody. See Figure 1.

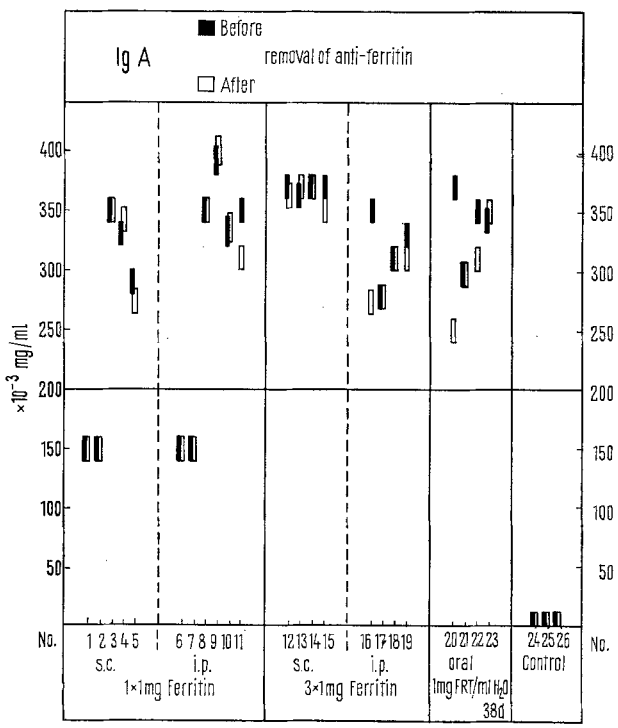

Fig. 4. Quantitative analysis of serum IgA before and after remova of specific anti-ferritin antibody. See Figure 1.

with anti-FRT specificity (Figure 4). The rise of 'non-anti-FRT' though small in comparison with that of 'non-anti-FRT' from other classes, was a constant feature of all but 4 immunized animals regardless of the route of administration of the antigen (Figure 4).

The data recorded here for IgG_1 and IgG_2 agree with previous reports of similar studies on conventional mice[7-8] and minimally contaminated mice[5] given ferritin by the parenteral route. In addition, specific antibody of the IgM and IgA types were found in some of the i.p. immunized animals. The data of SELL[9] and BAZIN[10] suggest that higher antibody titers might have been obtained in those mice receiving a single antigen dose, had longer intervals separated the bleeding from the immunizing injection.

The antibody response, in orally stimulated mice, being confined to the IgA class, further emphasizes the role of the IgA-producing lymphoid tissue of the gut in the immunological response to antigenic material present in the intestinal lumen.

This report also indicates that increases in immunoglobulins not reacting with the antigen frequently occur in all immunoglobulin classes, though they are proportionately most pronounced with IgM.

Résumé. Après injection de ferritine de cheval, des souris C_3H axéniques développèrent des anticorps circulants ainsi que des immunoglobulines sans affinité pour l'antigène dans chacune des classes: IgA, IgM, IgG_1, et IgG_2. Après immunisation orale, la réponse spécifique fut exclusivement du type IgA, et la réponse non-spécifique principalement du type IgG_2 et IgA.

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[7] W. F. BARTH, C. L. MCLAUGHLIN and J. L. FAHEY, J. Immun. *95*, 781 (1965).
[8] F. L. ADLER, J. Immun. *95*, 39 (1965).
[9] S. SELL, J. Immun. *95*, 300 (1965).
[10] H. BAZIN, Ann. Inst. Pasteur *112*, 162 (1967).
[11] Belgian American Educational Foundation Fellow.
[12] Staff Member of the Biology Division of Euratom, Euratom Publication No. 486.
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Anti-Hapten Antibodies in the Serum and Bronchial Secretions of Dogs Following Respiratory Tract Immunization

During the past few years considerable information has accumulated concerning the secretory immunologic system. It has been demonstrated that IgA is the predominant immunoglobulin in the secretions of man[1-3], rabbits[4,5] and dogs[6].

It now appears possible to selectively stimulate the respiratory secretion immunologic system in man and to obtain protection against certain viral infections[7,8]. Only a single report exists showing the local induction of secretory IgA antibody directed toward a defined antigenic determinant. These investigators found that anti-dinitrophenyl (DNP) antibody of the secretory IgA class can be elicited by local injection of the antigen into rabbit mammary tissue[9].

The purpose of this investigation was to stimulate the production of secretory anti-hapten antibody and if possible to specifically purify and characterize the antibody.

Materials and methods. 3 adult mongrel dogs were used for this study. Immunization and the collection of blood and bronchial secretions were done while the dogs were under sodium pentothal anesthesia. To obtain bronchial secretions, 10 ml of phosphate buffered saline was instilled via a bronchoscope and immediately removed by suction. Approximately 6 ml was recovered from each dog. The 3 washes were pooled, homogenized in a tissue grinder, centrifuged and concentrated to 3 ml. Serum samples were also pooled (equal volumes from each dog).

During a 12-week period the dogs received 3 intratracheal injections of 2.5 mg 2,4-dinitrophenylated keyhole limpit hemocyanin (DNP-KLH). 4 weeks later another intratracheal injection was given and DNP-KLH was administered by aerosol. Samples of blood and bronchial secretions were obtained 3 weeks later and an additional 10 mg DNP-KLH was given via a bronchoscope. Blood and bronchial washes were obtained 5 weeks following the final antigenic challenge.

Sera and bronchial washes were assayed for anti-DNP activity using a modified phage neutralization assay, employing DNP-T_4 bacteriophage conjugates[10]. The specificity of the neutralization reaction was determined by: (a) preincubating dilutions of selected samples with $2\times10^{-2}M$ ε-DNP-caproic acid for 1 h at 37°C and comparing the neutralizing capacity with serum and bronchial aliquots diluted with saline in place of the hapten inhibitor; (b) testing samples against unconjugated T_4 bacteriophage.

One immune serum pool was assayed in order to establish the kinetics of DNP-T_4 neutralization. The Figure shows that under the conditions of these experiments neutralization followed first-order kinetics for at least 10 min. Thus all samples were assayed using 10 min neutralization times. The potency of the various samples is expressed in terms of the rate constant for phage inactivation:

$$K = \frac{-1n\,(p/p_0)}{C\Delta T}$$

where p = number of plaques at time t; p_0 = number of

[1] W. B. CHODIRKER and T. B. TOMASI, Science *142*, 1080 (1963).
[2] J. S. REMINGTON, K. L. VOSTI, A. LIETZE and A. L. ZIMMERMAN, J. clin. Invest. *43*, 1613 (1964).
[3] T. B. TOMASI, E. M. TAN, A. SOLOMON and R. A. PRENDERGAST, J. exp. Med. *121*, 101 (1965).
[4] J. J. CEBRA and J. B. ROBBINS, J. Immun. *97*, 12 (1966).
[5] J. J. CEBRA and P. A. SMALL, Biochemistry *6*, 503 (1967).
[6] J. S. JOHNSON and J. H. VAUGHN, J. Immun. *98*, 923 (1967).
[7] R. H. WALDMAN, J. J. MANN and P. A. SMALL, J. Am. med. Ass. *207*, 520 (1969).
[8] L. P. TREMONTI, L. L. JUEY-SHIN and G. G. JACKSON, J. Immun. *101*, 572 (1968).
[9] R. J. GENCO and M. A. TAUBMAN, Nature *221*, 679 (1969).
[10] The DNP-T_4 conjugate used was kindly provided by Dr. O. MÄKELÄ. O. MÄKELÄ, Immunology *10*, 81 (1966).