

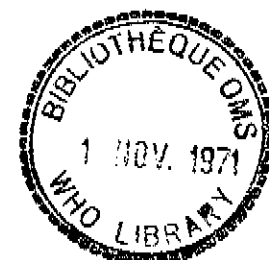


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IMMUNE ANTI-BACTERIAL MECHANISMS AT MUCOUS SURFACES

by

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Since we are to be mainly concerned with oral immunization against enteric infections, my remarks will be largely confined to considerations of intestinal immunity, though no doubt some of the points made will have a bearing on immunity at other mucous surfaces.

Even before we consider the special factors which bear on the immune reactivity and resistance of the intestinal tract, perhaps we should first decide whether immunological mechanisms are of prominent importance in resisting enteric infections. The best positive evidence is of the kind which shows that conventionally immunized people or animals have a much better chance of surviving attack by the specific parasite. An excellent example in man is provided by the trials of typhoid vaccine by Ashcroft et al. in children of Guyana (1967). One injection of acetone-dried killed vaccine gave 90 per cent. protection for up to seven years. This indicates that excellent immune resistance can be achieved by vaccination, but in spite of the fact that typhoid is an enteric disease in which the organisms enter the host by mouth there is no certainty from these studies that the immunity is conferred in the gastro-intestinal tract. It is conceivable, though unlikely, that the immunity could be operating at a later stage when the typhoid organisms have penetrated beyond the intestine into the blood stream, etc.

Perhaps a clearer example of antibody-mediated immunity is to be found with the neonatal (bacterial) enteric infections of man and animals. These can be caused by a wide variety of organisms but most commonly by E. coli; the specific bacterial types involved occur fairly widely but apparently without harm in adult animals. It seems likely to me that the specific serotypes are not themselves peculiarly virulent except in so far as they are the strains against which the new-born animal possesses negligible amounts of antibody, for a variety of reasons. At any rate, it seems clear from the studies of Lovell (1937) on calf scours that maternal transmission of specific antibody via colostrum is an extremely efficient way of preventing these devastating enteric infections in which the causative organisms are confined to the gut at least in the earlier stages.

Cholera studies also provide good support for the existence of specific intestinal immune mechanisms against enteric infection, since the excellent vaccine trials in recent years in India and Pakistan. Here we have a clear example in which the immune mechanisms, whatever they are, must be acting in the gut since the multiplication of the cholera vibrio during the disease takes place there exclusively. Strangely enough, in this infection a good correlation has been noted between protection and serum antibody levels as determined by

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a complement-mediated bactericidal test (Mosley, Benenson & Barui, 1968). It should be noted that this is in direct contrast with the early work of Burrows, Elliott & Havens (1947) using a rather unnatural cholera infection of guinea-pigs; they found that resistance following specific immunization was not correlated with serum antibody levels but showed some correlation with the amounts of intestinal antibody. In spite of their dubious data, Burrows and colleagues made the important point that with infections like cholera the quantities of copro-antibody were likely to be of determinant importance and that these were not necessarily related to the serum antibody levels.

The examples and considerations above establish an overwhelming case for an important protective role for specific antibody and possibly other immunological reactions in defending animals and man against intestinal parasites of many kinds. Some of this protection is expressed in and by the intestinal tract and we should consider how this may operate locally and what factors may alter the efficiency of these resistance mechanisms.

Because of the difficulties of measuring copro-antibody levels, the ideas of Burrows regarding the significance of these localized antibodies did not receive much factual support until the discovery of different antibody classes, IgM by Deutsch, Alberty & Gosting (1946) and IgA by Heremans & Schultze (1959). The existence of immunoglobulins of widely differing molecular weights made it quite clear that intestinal antibodies, even if derived from serum by simple filtration, would not have the same distribution between the different classes as occurred in the serum. During the past 10 years progress towards quantitation of copro-antibodies has been extremely rapid, mainly due to the application of the following techniques: (1) fluorescent antibody staining using antisera against the various immunoglobulin classes, which has allowed the precise localization and enumeration of the cells producing the three main immunoglobulins, and (2) the elegant and simple radial immuno-diffusion technique of Mancini, Carbonara & Heremans (1965) which has facilitated quantitative estimations of the three immunoglobulin classes in secretions obtained from many sites including the intestine.

With the fluorescent antibody staining methods, Crabbé & Heremans (1966) have shown that there are large numbers of cells producing antibodies in the lamina propria of the human intestine, and that cells producing IgA predominate over the other two, to a variable but considerable degree. Similar results have been found with all other animal species studied. Table 1 shows some data on this point collected from several sources, and one can see that the numbers of IgA cells often far exceed the combined total of the other two. Relatively few IgA forming cells can be found in other tissue of the body, such as the spleen or lymph nodes, but they have been seen in all mucosal surfaces examined - lungs, bladder, salivary glands, etc. Accepting that these large numbers of immunoglobulin forming cells in the lamina propria are releasing antibodies into the tissue fluids, one would expect that some of these antibodies would diffuse to the intestinal epithelial surface and that a proportion would reach the blood stream. This seems to be the case since Crabbé et al. (1969) have shown that, following oral immunization of mice with ferritin, IgA which appeared in the serum had originated from intestinal synthesis. This conclusion is greatly strengthened by the recent work of Bazin et al. (1971) who studied the effect of irradiation on the serum immunoglobulin levels of mice. If mice were totally irradiated there was a selective drop in their serum IgA level which did not occur if the intestine was shielded. The synthesis of IgA by the irradiated intestine was not greatly reduced so they concluded that irradiation caused an alteration in the proportions of the IgA diffusing into serum or intestine, in favour of the latter pathway. These and many of the quantitative results on immunoglobulin contents and distribution have been obtained using the valuable radial immuno-diffusion test. In Table 2 the published immunoglobulin compositions of intestinal juice from various animals have been assembled. In view of the IgA cell predominance, it is surprising to find from many of these studies that the IgM levels are proportionately higher than would be expected if all the IgM had been derived from the specific cells of the lamina propria.

Since we accept that most of the intestinal IgA and indeed that found elsewhere in the body is derived from enteric synthesis, it follows that some of the IgM which appears in the intestinal juice in amounts greater than expected has probably been synthesised elsewhere. This would be an easily acceptable explanation for the excess IgG since this is known to equilibrate between serum and intestine, but IgM has been regarded as immobile and non-diffusible due to its higher molecular weight.

In brief summary of the excellent work done by the Heremans group on intestinal IgA formation, the following points already made by Heremans seem particularly important and acceptable to me:

- (1) The intestinal lamina propria is the major source of all the IgA in the body.
- (2) This local synthesis can be induced by antigens given by mouth; with efficiencies which vary with certain characters of the antigen, such as their particulate nature.
- (3) The ratio of IgA concentration in secretion to that in serum is very much higher than those for the other two immunoglobulins.

This supports the thesis that the general origin of IgA is quite different from the sources of IgM and IgG.

How are we to interpret the three main pieces of factual data above in relation to the mechanisms of enteric immunity and its stimulation by antigenic materials? At the moment it seems that there is insufficient good information on which to base predictions with any degree of certainty and I will discuss some of the areas of doubt in the following.

A. Transport of immunoglobulin classes

In Table 2 we see that there are quite large amounts of IgM in the intestinal fluid of man and mouse and probably in other animals, although there are relatively few IgM forming cells in the lamina propria as revealed by fluorescent antibody staining. Since these distributions of cells and immunoglobulin classes are based on good data, we obviously have a problem in understanding how such large amounts of IgM arrive in the intestine. It seems that this must be either by transport from the serum or following local synthesis in the lamina propria which would involve a lesser transport problem. Two facts favour transport: firstly, the paucity of IgM forming cells in the intestines of a wide variety of animals and, secondly, the low ratio of IgM in secretions compared to that in serum. This must be contrasted with the very high secretion/serum ratio for IgA which is an important distinguishing feature of that immunoglobulin as pointed out by Vaerman (1970).

If IgM arrives in the intestine by transport - contrary to all "classical" expectations - then we need information about the efficiency of this process. It would be preferable also if the transport studies used an antibacterial functional marker, for obvious reasons. Such studies have not been done, and what information there is to indicate that antibody transport from serum to intestine exists is unrelated to antibody class (Batty & Bullen, 1961).

We have made some attempts to study immunoglobulin transport using ¹³¹I-labelled, purified classes of rabbit antibody, by following transport into ligated loops of rabbit intestine during the development of permeability and inflammation following large doses of cholera toxin. Considerable quantities of each of the three classes were transported varying from 1.0 per cent. with IgM to 2.6 per cent. with IgG, expressed as percentage of the serum level. Our studies only apply under conditions of inflammation which of course may be present during enteric infections (Wernet et al., 1971). They have the disadvantage of using a non-biological marker which was necessitated by the low biological activities of our immunoglobulin preparations. It would be preferable to repeat these studies using biological activity to follow transport in a model such as the canine Thiry-Vella intestinal loop.

B. Protection tests with each class of antibody

For the reasons given in the introduction, we must accept that specific enteric immunity exists and that it is mainly antibody-mediated since it can be transferred efficiently from mother to offspring via the milk.

It has been claimed that IgA is more resistant to proteolytic digestion than other immunoglobulins and therefore is well adapted to be the ideal functional antibody in the intestine. Until we have some better knowledge as to how antibodies may work in the gut, this is not a persuasive argument. If, for instance, antibodies act only for the short time that they are actually in contact with or very close to the intestinal epithelium, then what happens to them subsequently when they have been released into the lumen may be quite irrelevant to their action.

Some simple and interesting experiments have been published by Freter (1970) aimed at understanding the protective effects of copro-antibodies. He counted the numbers of Vibrio cholerae firmly adsorbed to pieces of rabbit intestine after exposure to suspensions of the organisms in the presence or absence of specific antibody. Surprisingly, he found that in presence of antibody far fewer organisms were adsorbed. Initially he suggested that antibody prevented the sticking of bacteria to the intestinal epithelium and that this could be a direct protective mechanism. Further experiments caused him to modify his views and to suggest that, following exposure to antibody, the vibrios were rapidly killed by the intestinal epithelium following adsorption, presumably by one or other of the two well-known antibacterial mechanisms.

We have repeated and elaborated Freter's experiments and can confirm that exposure to antibody does actually diminish the numbers of Vibrio cholerae attached to the rabbit or mouse intestinal surface. By using ³²P-labelled organisms we could show that the effect was not due to subsequent killing after adsorption but to an initial reduction in the numbers adsorbing. No evidence of killing following adsorption has been found, but organisms adsorbed to intestine in the absence of antibody were resistant to exposure to very large excesses of antibody and complement or to terramycin, presumably due to inaccessibility. The smaller numbers of organisms attached in presence of antibody were killed by subsequent treatment with antibody and complement or by terramycin, so they must be presumed to be attached more superficially. These findings (Chaicumpa, W. & Rowley, D. (1970) personal observations) and the demonstration below that antibodies cause a sharp reduction in numbers of viable bacteria in the gut indicated to us that this was a misleading model for enteric immunity and we have abandoned it.

Whilst there is good evidence that antibodies protect against enteric infections, there has not been a clear indication that this results directly in a reduction of bacterial numbers. Using the baby mouse model for cholera first described by Ujiye et al. (1968), we have found in agreement with them that opsonisation of the vibrios with specific antibody, passive administration of specific antibody via the peritoneum or suckling the baby mice on immunized mothers after infection will all lead to great reductions in mortality. This protection is accompanied by dramatic reductions in the numbers of vibrios in the baby mice intestines. There are no vibrios elsewhere in the infant mice and the reduction is due to an intra-intestinal bactericidal effect which is not affected by blockage of the anus (Fig. 1) (Chaicumpa & Rowley, 1971).

It is of interest to note that maternal mice can be effectively immunized orally by either living or killed Vibrio cholerae and can then transmit their resistance to six-day-old infected young. This animal model offers a convenient assay for both the protective antibodies and the particular bacterial antigens involved. We reported recently that at least two antigens exist in V. cholerae which serve as initial substrates for the antibody-mediated bactericidal reaction. One of these belonged to the well-known class of lipopolysaccharides found generally in the cell walls of gram-negative bacteria and whose determinant antigenic groups are due to polysaccharide structures. The other antigen was a protein which normally existed firmly attached to the lipopolysaccharide structure but which could be split from it

by extraction into phenol. We have now tested the protective effects of antibodies against the polysaccharide determinants of the lipopolysaccharide compared to those against the protein component, using the baby mouse cholera model. Table 3 shows that on a weight basis the anti-protein antibodies are at least as protective as the anti-polysaccharide ones and that both of these are much more effective than the anti-cholera toxin - kindly supplied by Dr R. Finkelstein (Neoh & Rowley, 1971). This work must now be extended to cover the protective abilities of the different classes of antibody.

C. The relative antibacterial efficiencies of IgA, IgM and IgG

The proportions of the three immunoglobulins in the intestinal juice (Table 2) make it necessary to recognize that IgM is a strong candidate for functional copro-antibody and that even IgG cannot be lightly dismissed from our considerations in this regard. Of course, the fact that in mice and man there are rather comparable amounts of each immunoglobulin class tells us nothing about their contributions to immunological defence. We need information on the specific antibody contents within the classes. In the few cases where this is available (usually measured by a non-functional test such as agglutination) the specific activity has been found to some extent in all three but mainly in IgM (Bourne, Honour & Pickup, 1971). Because of the known differences in haemagglutinating, haemolytic and opsonising efficiencies of IgM and IgG (Rowley & Turner, 1966), the significance of these assays is uncertain. We need a clear answer to the question whether a milligram of specific IgM in the intestine is more effectively antibacterial than a milligram of specific IgG or IgA.

Broadly speaking, there are only two known functional antibacterial attributes of antibody. The one involves complement and results in a direct bactericidal effect against many gram-negative bacteria, and particularly those involved in enteric infections. The second is the more generally applicable one by which specific antibody attaches to bacteria and renders them susceptible to phagocytosis by leucocytes of various kinds. A third attribute by which specific antibodies may neutralize bacterial toxins, though of course of great importance, is not antibacterial in the sense that the parent organisms which produce the toxins are not directly affected. It is important to compare the immunoglobulin classes by tests which have some relevance to these two main functional parameters whilst bearing in mind that there could conceivably be some entirely new functional pathway yet unknown.

Two groups have tried to provide this data with completely conflicting results. Eddie, Schulkind & Robbins (1971) purified specific Salmonellae antibodies of each class from rabbit intestinal juice and colostrum, then compared their activities on a molar basis by the direct C'-mediated bactericidal reaction and by their ability to promote intravenous clearance of the specific organism. Their results were clear: IgM was about 1000 times more active than IgG on a molar basis in both these tests, which is in good agreement with the results of others (Rowley & Turner, 1966). On the other hand, IgA was devoid of significant activity in either test although possessing quite high agglutinating powers. From their results, IgA seems functionally inert as an antibacterial antibody.

My own group has tried to provide answers to the same question (Knop, Brey, Wernet & Rowley, 1971). We purified the colostral immunoglobulins from pigs and rabbits immunized against E. coli and compared them by the direct bactericidal test and by a phagocytic test using the mouse peritoneum as a source of phagocytic cells. Whilst we also found that IgA lacked activity in the direct bactericidal test, in our hands the IgA was very active as an opsonin (Table 4).

We must concede that Robbins and his group would be more skilled at protein purification than we are, and indeed we did find impurity in our best preparations of IgA. Yet it is impossible to account for the high activity of our IgA preparations in terms of IgM or IgG impurity since these were themselves so much less active, per haemagglutinating unit. We are currently repeating this work in Adelaide and extending our investigations to cover dog and mouse immunoglobulins using Vibrio cholerae as the antigen.

D. Stimulation of specific antibody formation in particular immunoglobulin classes

Before long we are certain to know which class of antibody offers the most effective protection against enteric infection and our problem will then be how to direct antibody synthesis into that particular class. If this class happens to be IgA, it seems likely that antigens given by mouth will be useful. As mentioned earlier, particulate antigens seem more effective when given orally than do smaller soluble antigens. Could this indicate that antigens adsorbed on aluminium hydroxide might be more stimulatory or that adjuvants might operate in the intestinal antibody response? There could be a fertile field for the lipopolysaccharide chemists here.

The possibility that IgA is an effective antibody is a real one and therefore the prospects for renewed interest in oral immunization seem good to me. The advantages are obvious, being particularly ones of convenience and reduced concern for sterility or toxicity. The ease of administration offered by oral vaccines could itself be a disadvantage by removing dosage from medical supervision, but this seems to have been well-controlled in the oral polio vaccine programme. Given encouraging answers to some of the questions I have posed above, I believe there may be great progress with oral immunization against some enteric infections.

REFERENCES

- Ashcroft, M. T., Singh, B., Nicholson, C. C., Ritchie, J. M., Sobryan, E. & Williams, F. (1967) Lancet, 2, 1056
- Batty, I. & Bullen, J. T. (1961) J. Path. Bact., 81, 447
- Bazin, H., Maldague, P., Schonne, E., Crabbé, P. A., Bauldon, H. & Heremans, J. F. (1971) Immunology, 20, 571
- Bourne, F. J., Honour, J. W. & Pickup, Jill. (1971) Immunology, 20, 433
- Bull, D. M., Bienenstock, J. & Tomasi, T. B. (1971) Gastroenterology, 60, 370
- Burrows, W., Elliott, N. E. & Havens, I. (1947) J. infect. Dis., 81, 261
- Chaicumpa, W. & Rowley, D. (1971) J. infect. Dis. (In press)
- Crabbé, P. A. & Heremans, J. F. (1966) Gastroenterology, 51, 305
- Crabbé, P. A., Nash, D. R., Bazin, H., Eyssen, H. & Heremans, J. F. (1969) J. exp. Med., 130, 723
- Deutsch, H. F., Alberty, R. A. & Gosting, L. J. (1946) J. biol. Chem., 165, 21
- Eddie, D. S., Schulkind, M. L. & Robbins, J. B. (1971) J. Immunol., 106, 181
- Freter, R. (1970) Infect. Immun., 2, 556
- Girard, J. P. & Kalbermatten, A. (1970) Europ. J. clin. Invest., 1, 188
- Heremans, J. F., Heremans, M. T. & Schultze, H. E. (1959) Clin. chim. Acta, 4, 96
- Knop, J., Breu, H., Wenet, P. & Rowley, D. (1971) Aust. J. exp. Biol. med. Sci. (In press)
- Lovell, R. (1937) J. Path. Bact., 44, 125
- Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) Immunochemistry, 2, 235
- Mosley, W. H., Benenson, A. S. & Barui, R. (1968) Bull. Wld Hlth Org., 38, 327
- Neh, S. H. & Rowley, D. (1971) J. infect. Dis. (In press)
- Neh, S. H. & Rowley, D. (1971) Aust. J. exp. Biol. med. Sci. (In press)
- Rowley, D. & Turner, K. J. (1966) Nature, 210, 496
- Ujiye, A., Nakatomi, M., Utsunomiya, A., Mitsui, K., Sogame, S., Iwanaga, M. & Kobari, K. (1968) Trop. Med. (Nagasaki), 10, 65
- Vaerman, J. P. (1970) Thesis, University of Louvain
- Wenet, P., Breu, H., Knop, J. & Rowley, D. (1971) J. infect. Dis., 124, 223

TABLE 1. THE FREQUENCY RATIOS OF ANTIBODY-FORMING CELLS IN THE GUT

	IgA	IgG	IgM	
Human duodenum	22	1	3.3	Crabbé and Heremans (1966)
Human colon	23	1	1	"
Mouse ileum	150	10	1	Bazin et al. (1971)
Dog intestine (Average)	2	1	1	Vaerman (1970)

TABLE 2. THE CONCENTRATIONS OF IMMUNOGLOBULINS IN INTESTINAL FLUID

	IgA	IgG	IgM	
Human adults	31 mg/100 ml	10	20	Girard and
Human infants	13 "	4	21	Kalbermatten (1970)
Rabbits	15 "	10	2.4	Eddie, Schulkind and Robbins (1971)
Mice	172 "	99	524	Bazin et al. (1971)
Pig	700 "	110	20	Bourne, Honour & Pickup (1971)
Human	30 "	30	-	Bull et al. (1971)

TABLE 3. PROTECTIVE EFFECT OF ANTIBODIES IN THE INFANT MOUSE MODEL*

Antiserum	μ g Antibody/ml	Haemagglutinating titre	Vibriocidal titre	PD ₅₀ (μ g/antibody) vs. 2000 LD ₅₀ challenge
Live 569B	1,800	1/3,200	1/620,000	2.4 μ g
Anti-protein	320	<1/4	1/16,000	1.9 μ g
Anti-cholera- gen (R. A. Finkelstein)	>2,000	1/64	<1/20	>40 μ g

* Data from Neoh, S. H. & Rowley, D. (1971).

TABLE 4. BIOLOGICAL ACTIVITY OF PIG COLOSTRAL IMMUNOGLOBULIN CLASSES

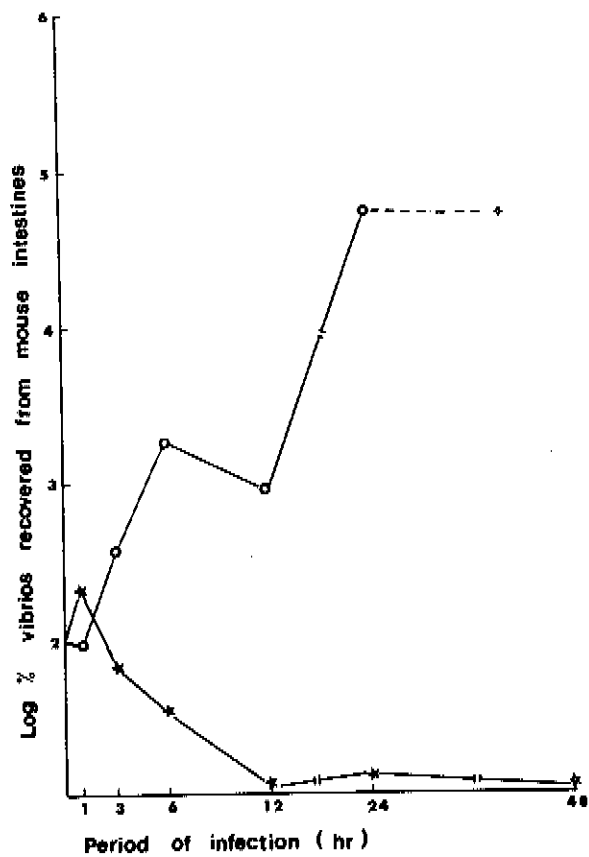
Ig	H. A.	B.	I. K.
CLASS	per mg PROTEIN		
IgM	2500	10000	70000
IgG	40	100	2000
IgA	20	11	50000

H. A. = 1/haemagglutination titre

I. K. = titre giving 90% intracellular killing

B. = titre giving 50% bactericidal activity with C'

FIGURE 1.



★ Vibrio cholerae with antibody.

○ Vibrio cholerae without antibody.