Immunology 1983 **48** 687

IgA antibodies in the bile of rats

III. THE ROLE OF INTRATHORACIC LYMPH NODES AND THE MIGRATION PATTERN OF THEIR BLAST CELLS

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Accepted for publication 9 September 1982

Summary. Eight weeks after rats had had their mesenteric lymph nodes (MLN) removed surgically, they were found to be still able to generate substantial titres of biliary IgA-antibodies after antigens were injected into their Peyer's patches. This suggested that systemically significant IgA production could be induced in extra-abdominal lymphoid tissue. It was found that the intrathoracic lymph nodes (ITLN) were an important source of IgA production. These nodes could be stimulated to produce biliary antibody by introducing antigen either into the peritoneal cavity or directly into the thorax. Cells forming IgA were identified in the ITLNs by haemolytic plaque assays and immunoperoxidase techniques. In spite of this, immunoblasts obtained from the ITLNs, and labelled with ^{125}IUdR did not localize in the gut after i.v. injection to anywhere near the extent that immunoblasts from the MLN did. Instead they seemed to have a predilection for localizing in the lungs.

INTRODUCTION

The experimental antigenic stimulation of the gut-associated lymphoid tissue (GALT) of rats results in the production of specific antibodies of the IgA class. Unlike other classes of Ig, these antibodies are secreted abundantly in the bile (Hall, Orlans, Reynolds, Dean, Peppard, Gyure & Hobbs, 1979; Andrew & Hall, 1982a and b). In such experiments the bulk of the antibody-forming cells are situated in the mesenteric lymph nodes (MLN) and the lamina propria of the intestine (Andrew & Hall, 1982a), but under physiological conditions many cells making IgA can also be found in the mucosae and regional lymphoid tissue of the respiratory and genito-urinary tracts. Indeed Bienenstock has coined the term MALT (mucous-associated lymphoid tissue) to suggest the functional integration of the anatomically dispersed lymphoid tissues which mediate the production of the antibodies found in various external secretions (Bienenstock & Befus, 1980).

If it is true that lymphoid tissue other than the GALT is quantitatively important in humoral responses involving IgA-antibodies, it should be possible by the antigenic stimulation of, say, the intrathoracic lymphoid tissue, to induce the appearance of significant titres of specific antibodies in the bile of rats. This paper records the experimental test of this proposition.

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0019-2805/83/0400-0687$02.00

MATERIALS AND METHODS

Animals

Male Wistar rats (Wistar/PCbi) weighing approxima-

tely 200 g were taken from our own, inbred, barrier-maintained colony as required. For reasons of economy it was necessary occasionally to use female Hooded rats.

Surgical procedures

The induction of anaesthesia, injection of antigens into Peyer's patches (PP), cannulation of the common bile duct, restraint of prepared animals in Bollman cages and the collection of bile and serum have been described (Hall *et al.*, 1979) as has the excision of mesenteric nodes (Hall, Hopkins & Orlans, 1977).

Antigenic stimulation of the mediastinal nodes was achieved by injecting 0·10 ml of a suspension of antigen into the thoracic cavity. The abdomen was opened, as for the injection of Peyer's patches, and the viscera were retracted to expose the under surface of the left side of the diaphragm through which a 27 guage needle, mounted on a tuberculin syringe, was inserted directly into the thorax. With practice, this could be done without causing a significant pneumothorax or damaging the lung.

Antigens etc.

Suspensions of killed, dyed *Brucella abortus* organisms (approximately 10^{10} per ml) were obtained from the Wellcome Research Laboratories, Beckenham, Kent. Sheep red blood cells (SRBC) were isolated from heparinized blood obtained from sheep by direct percutaneous jugulo-venous puncture as required.

Radioactive colloidal gold (^{198}Au injection BP) was obtained from the Radiochemical Centre, Amersham, and its emission assayed in a γ spectrometer.

Lymphatics and lymph nodes were visualized with the aid of a lymphography dye, Patent Blue V, obtained as a sterile, 2·5% solution from May and Baker Ltd., Dagenham.

Antibody titrations

Agglutinating antibodies in blood and bile were titrated by standard methods using doubling dilutions in 0·05–0·10 ml systems, as described (Hall *et al.*, 1979).

Plaque-forming cell assays and determination of antibody isotype

Lymph-node cells making antibodies against SRBC were detected by plaque-formation in suspensions after the method of Cunningham & Szenberg (1968). Fresh guinea-pig serum was used as a source of complement. The anti-γ and anti-α reagents necessary for developing plaques mediated by IgG or IgA antibodies were made from hyperimmune rabbit or goat antisera by affinity chromatography.

Labelling of blast cells with [^{125}I]UdR

Suspensions of lymph-node cells were incubated *in vitro* with ^{125}iodo-deoxy-uridine (^{125}IUdR) at a concentration of 0·5 μCi (19 kilobecquerels) per ml as described (Halstead & Hall, 1972). This procedure labels cells that are synthesizing DNA, i.e. the blast cells, but not small lymphocytes. The labelled cells were injected i.v. into syngeneic recipients which were killed 20 hr later. Selected organs were excised, loaded into counting vials and the γ-emission counted in a 'Wilj gamma counter 2001'.

Cell counts

White cells were counted, after appropriate dilution in 1·5% acetic acid, in a Neubauer haemocytometer.

Histology

Rat lymph nodes were fixed in Bouin's solution and sections were prepared by standard methods. The isotype of immunoglobulin in lymph node cells was demonstrated by treating unstained sections with immunoperoxidase antiglobulin reagents, using a sandwich technique (Hall *et al.*, 1977).

RESULTS

Effect of excising mesenteric nodes

Previous experiments (Andrew & Hall, 1982a) had shown that after the injection of antigens in the PP of rats most IgA-forming cells were found in the mesenteric nodes. Accordingly, the mesenteric nodes were excised from fourteen rats; 2 months later, when the lymphatic vessels had regenerated, 5×10^8 killed *Br. abortus* organisms were injected into the PP of each rat. The resulting titres of specific antibody in the bile were monitored and compared with those in the bile of normal control rats which had received identical injections of antigen. The results are shown in Fig. 1. It can be seen that although the rats that had had their mesenteric nodes removed produced significantly less biliary IgA-antibody than the controls, they were still able to generate substantial amounts. Clearly, the synthesis of IgA had been proceeding in sites other than the mesenteric nodes.

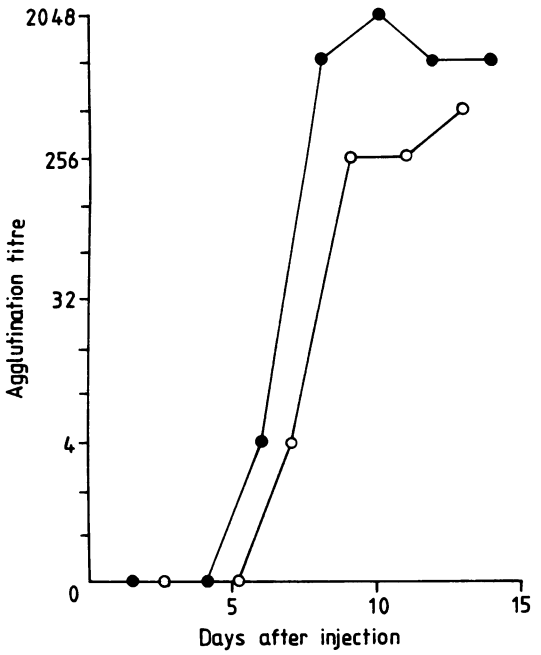

Figure 1. The titres of specific antibody in the bile of normal rats (●——●), and in the bile of rats that had had the mesenteric lymph nodes excised (○——○), after both groups had been given injections of 10^9 killed *Br. abortus* into their Peyer's patches at time zero. Each point represents the results obtained by pooling equal volumes of bile from two rats.

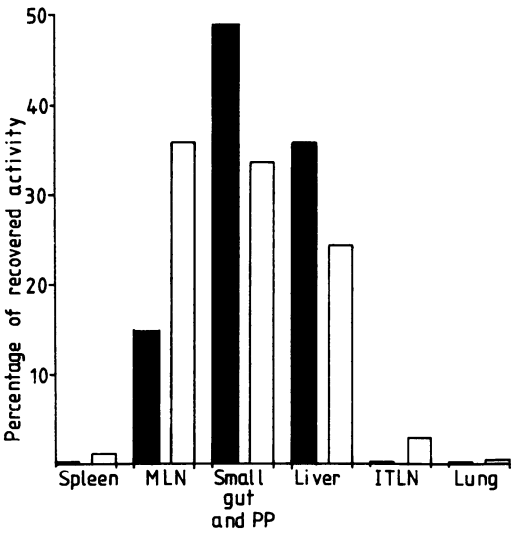

Figure 2. The recoveries of radioactivity in various organs of rats at 2 hr (blocked histogram) and 24 hr (clear histogram) after they had been given injections of radioactive colloidal gold into their Peyer's patches.

Uptake of injected material by intrathoracic lymph nodes (ITLN)

The postmortem examination of rats that had received injections of antigens into their PP sometimes revealed that the paratracheal and parathymic nodes in the cephalic part of the mediastinum had undergone reactive hyperplasia, and were more obvious than in normal rats, where they tend to be inconspicuous. We reasoned that, after antigens had been injected into the PP, some leaked back intraperitoneally and were absorped by the diaphragmatic lymphatics, which drain the peritoneal cavity (Yoffey & Courtice, 1970), and conveyed to the ITLN. This was confirmed by injecting lymphography dye into the peritoneal cavity of some normal rats, which were killed a few minutes afterwards. The lymphatics draining from the pleural surface of the diaphragm could be seen to be filled with dye and to communicate with the parathymic and paratracheal lymph-nodes, which consequently became stained green. In order to show directly that particulate material injected into the PP could leak back and, via the peritoneum, reach the ITLN, five rats were given an injection of radioactive colloidal gold into their PP. They were killed either 2 or 24 hr later and the amount of radioactivity in various organs was assayed. The results are shown in Fig. 2. It can be seen that, although much of the dose was retained in the MLN and the injection sites in the small gut, a significant percentage had reached the ITLN by 24 hr. However, because these are relatively small structures, their specific radioactivities (c.p.s./g wet weight) were high and averaged $1{\cdot}3 \times 10^5$, compared with $5{\cdot}6 \times 10^5$ for the MLN and $4{\cdot}6 \times 10^3$ for the liver and spleen. It was concluded that, in the analogous situation of an injection of particulate antigen, the concentration of antigen in the ITLN would be able to initiate an immune response of sufficient magnitude to explain the results shown in Fig. 1.

Antigenic stimulation of intrathoracic lymph nodes

Fifteen rats received intrathoracic (IT) injections of 0·05 ml packed SRBC together with 0·05 ml saline containing 10^8 *Br. abortus* organisms. At intervals thereafter paired samples of blood and bile were obtained from each animal and the agglutinating titres

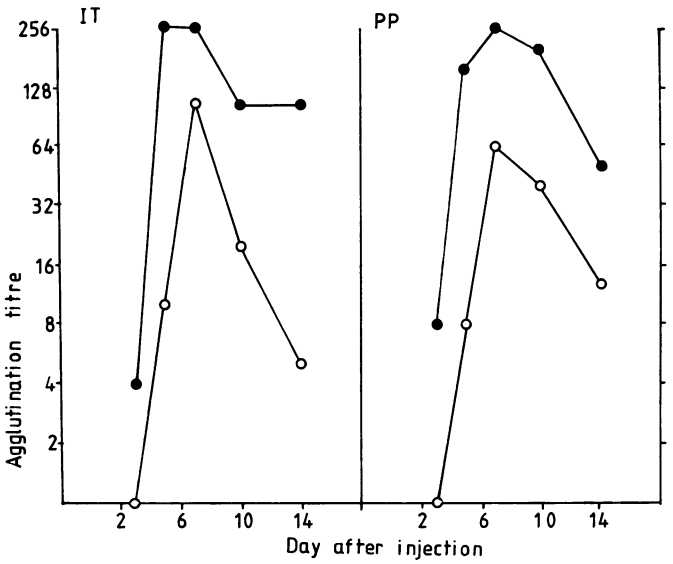

Figure 3. The titres of specific antibodies in the blood (●——●) and bile (○——○) of rats that had been given an injection of 0·05 ml packed SRBC and 10^8 killed *Br. abortus* organisms either into the Peyer's patches (PP) or into the thorax (IT) at time zero. The results shown refer to *Br. abortus* but similar results were obtained when the bile and serum were assayed for antibodies to SRBC. Each point represents the result obtained by pooling equal volumes of either bile or serum from three rats.

of specific antibodies were determined. Fifteen control rats received injections of the same doses of SRBC and *Br. abortus* into their PP.

The results are shown in Fig. 3. It is clear that the titres of biliary (IgA) and serum (IgG and IgM) antibodies were essentially the same in the IT and control (PP) immunized rats. In absolute terms all the titres were somewhat lower than previously published results (Andrew & Hall, 1982a) but this was because the female Hooded rats which had to be used are generally poorer responders than the male Wistar rats that were temporarily unavailable.

It seemed certain that the IT dose of antigen impinged principally on the ITLN, because when the rats were killed these nodes were seen to be markedly enlarged, and in those that had received dyed *Br. abortus* organisms, the blue colouration of the nodes was immediately apparent.

Isotypes of antibody produced by intrathoracic lymph nodes

In order to show directly that the ITLN could generate IgA-forming cells, suspensions of cells were prepared from the nodes of the above rats that had been immunized with SRBC. These suspensions were

Table 1. Numbers and isotype of the PFC in the MLN and ITLN of animals that had been given injections of 0·05 ml of packed SRBC into either the Peyer's patches or the thorax

Route of immunization	Day after injection	No. of PFC/10^6 lymphoid cells ITLN α	ITLN γ	ITLN μ	MLN α	MLN γ	MLN μ
IT	5	2787	2409	6839	4	1	5
	7	478	3579	2709	15	16	23
PP	5	1270	1025	4704	332	260	1006
	7	132	1336	978	2950	7150	503

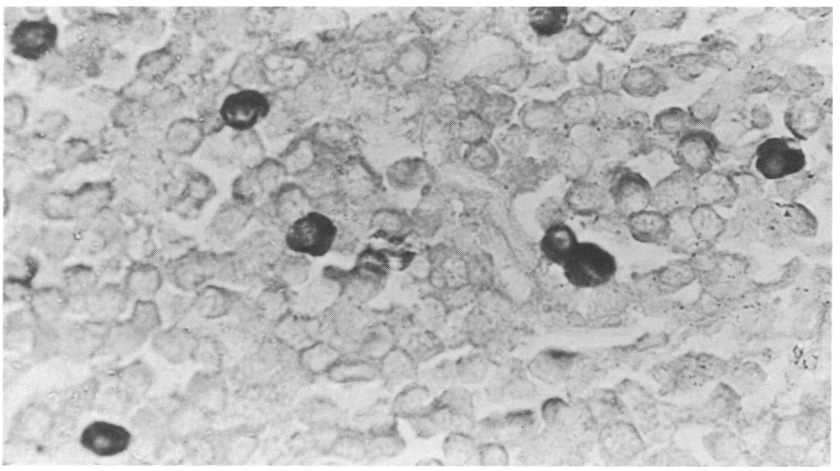

Figure 4. Photomicrograph (approx. × 500) of a section cut from a mediastinal lymph node of a rat. The section was treated with a specifically purified rabbit anti-rat α reagent and then with a goat anti-rabbit F(ab′)$_2$–peroxidase conjugate. After treatment with diamino-benzidene reagent the IgA-forming cells were seen to be slightly sparser than in mesenteric nodes but very much more frequent than in peripheral somatic nodes where few were seen.

assayed for plaque-forming cells (PFC) and the isotype of the Ig involved was determined. The results, shown in Table 1, make it clear that after either PP or IT immunization, significant numbers of IgA-PFC appear in the ITLN. A crude overall average of the results showed that some 15% of the plaques formed by ITLN cells involved IgA compared with 25% for the MLN cells.

The presence of IgA-forming cells in the ITLN was demonstrated also by applying immunoperoxidase reagents to histological sections (Fig. 4).

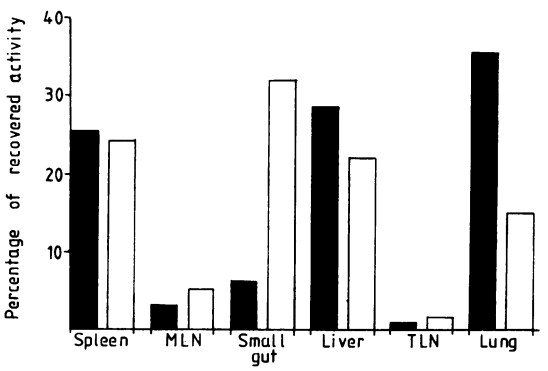

Figure 5. The distribution of radioactivity recovered in various organs of two groups of seven rats 20 hr after they had received i.v. injections of [^{125}I]UdR-labelled blasts from either the intrathoracic lymph nodes (blocked histogram) or the mesenteric lymph nodes (clear histogram).

Migration pattern of immunoblasts from ITLN

To determine how immunoblasts released from the ITLN might become distributed *in vivo*, suspensions of cells were prepared from those nodes 5 days after they had received an antigenic stimulus and been labelled *in vitro* with [^{125}I]UdR. The labelled cells, from twenty donors, were washed and injected i.v. into seven normal, syngeneic recipients. Twenty hours later the recipients were killed and the distribution of the labelled cells in selected organs were determined by the radioassay of their γ emission. The results were compared with those of seven control recipient rats where the labelled cells, obtained from MLNs, have a well documented pattern of distribution (Fig. 5). The salient finding was that whereas the blast cells from the MLN went, as expected, mainly to the gut, the blast cells from the ITLN were found mainly in the lung. Similar proportions of labelled cells from either source entered the spleen and liver.

DISCUSSION

The ITLN in the mediastinum are a heterogeneous group and their exact anatomical location and lymphatic connections vary widely between individuals and between species (Yoffey & Courtice, 1970). In general, they receive lymph from the lungs, pleura and mediastinal structures as well as from the peritoneum,

and their efferent ducts ultimately enter the thoracic duct or right lymph duct. It has been known for years that particulate material placed in the peritoneal cavity soon reaches the mediastinal lymphatics (Florey, 1927; Higgins, Beaver & Lemon, 1930), and it is clear from the present study that the deliberate or inadvertent introduction of antigenic material into the peritoneal cavity is likely to stimulate the ITLN. It is also clear that this stimulation leads to the production of IgA-antibodies in amounts that are sufficient to endow the bile with titres that are comparable to those obtained by the direct stimulation of the GALT. Indeed, it is likely that experimental stimulation of the GALT must often entail the inadvertent stimulation of the ITLN. All the same, it would not be correct to assert that these nodes are uniquely responsible for extra-abdominal IgA production. Significant IgA production can occur in the spleen, for example (Andrew & Hall, 1982a).

However, while it is easy to understand the apparent logic of producing biliary IgA-antibodies in response to antigens that impinge on the GALT (Hall & Andrew, 1980), it is less easy to see the purpose of producing potential biliary antibodies in the thorax. In the intact animal the IgA-antibodies produced in the ITLN would have little chance to reach the mucosa of the respiratory tract. They would be conveyed by the lymph to the blood and would be immediately extracted by the liver and secreted in the bile (Reynolds, Gyure, Andrew & Hall, 1980). In so far as microbial pathogens causing chest infections may be coughed up and swallowed the provision of specific biliary antibodies might serve to protect the gut, but the importance of such a mechanism has yet to be demonstrated. Perhaps the IgA-forming cells in the ITLN are visiting cells rather than strictly endogenous. They may be derived ultimately from the overflow of IgA-producing lymphoid tissue that is dispersed beneath the tracheo-bronchial mucosa and in the parenchyma of the lung. Cells from these sites could easily travel to the ITLN via the afferent lymph but there are no studies of peripheral lymph from the lungs to confirm or refute this. It is known, however, that the ITLN generate specific antibody-forming cells in response to antigens from the lungs (Bice, Harris & Muggenberg, 1980).

The propensity of immunoblasts from thoracic (i.e. intestinal) duct lymph or from MLNs to localize in the lamina propria of the gut is well documented and has been reviewed (Bienenstock & Befus, 1980). It is now generally agreed that neither local concentrations of antigens (Halstead & Hall, 1972) nor the isotype of the intracellular or surface Ig associated with the immunoblasts (Hall *et al.*, 1977) play any primary role in guiding them to their destination. This latter point is emphasized by the results of the present study. The cell suspensions prepared from the ITLN contained proportions of IgA-forming blast cells, which were not greatly less than those of suspensions prepared from MLN. In spite of this the migration patterns of the two populations were dissimilar, and the cells from the ITLN went to the lung while those from the MLN went to the gut. However, the facile explanation that some of the cells derived from ITLN are endowed with the ability to return to their region of origin cannot be accepted without serious reservations. Many types of cell that are injected i.v. are retained in the capillary bed of the lungs, albeit temporarily (Hall, Parry & Smith, 1972; Hall *et al.*, 1977), and the present experiments cannot wholly distinguish between cells that were retained in the lungs as a result of experimental artefact and cells that were retained by genuine immuno-physiological mechanisms. Nevertheless, immunoblasts obtained by collecting lymph efferent from the mediastinal nodes of unanaesthetised sheep, and which have undergone minimal trauma, show the same propensity to localize in the lungs (Hall & Spencer, unpublished observations), and we believe, at the moment, that the phenomenon has some biological significance.

ACKNOWLEDGMENTS

This work was supported by programme grants from the Medical Research Council and Cancer Research Campaign. We thank Drs Eva Orlans and Jane Peppard for providing the specifically purified anti-rat immunoglobulin reagents.

REFERENCES

ANDREW E. & HALL J.G. (1982a) IgA antibodies in the bile of rats. I. Some characteristics of the primary response. *Immunology*, **45**, 169.

ANDREW E. & HALL J.G. (1982b) IgA antibodies in the bile of rats. II. Evidence for immunological memory in secretory immunity. *Immunology*, **45**, 176.

BICE D.E., HARRIS D.L. & MUGGENBERG B.A. (1980) Regional immunologic responses following localized deposition of antigen in the lung. *Exp. Lung Res.* **1**, 33.

BIENENSTOCK J. & BEFUS A.D. (1980) Mucosal Immunity. *Immunology*, **41**, 249.

CUNNINGHAM A.J. & SZENBERG A. (1968) Further experi-

ments in plaque technique for detecting single antibody-forming cells. *Immunology*, **14,** 599.

FLOREY H.W. (1927) Reactions of, and absorption by, lymphatics, with special reference to those of the diaphragm. *Br. J. exp. Path.* **8,** 479.

HALL J.G. & ANDREW E. (1980) Biliglobulin: a new look at IgA. *Immunology Today*, **1,** 100.

HALL J.G., HOPKINS J. & ORLANS E. (1977) Studies on the lymphocytes of sheep. III. Destination of lymph-borne immunoblasts according to their tissue of origin. *Eur. J. Immunol.* **7,** 30.

HALL J.G., ORLANS E., REYNOLDS J., DEAN C., PEPPARD J., GYURE L. & HOBBS S. (1979) Specific antibodies of the IgA class in the bile of rats. *Int. Archs Allergy appl. Immunol.* **59,** 75.

HALL J.G., PARRY D.M. & SMITH M.E. (1972) The distribution and differentiation of lymph-borne immunoblasts after intravenous injection into syngeneic recipients. *Cell. Tissue Kinet.* **5,** 269.

HALSTEAD T.E. & HALL T.G. (1972) Homing of lymph-borne immunoblasts to the small gut of neonatal rats. *Transplantation*, **14,** 339.

HIGGINS G.M., BEAVER M.G. & LEMON W.S. (1930) Phrenic neurectomy and peritoneal absorption. *Am. J. Anat.* **45,** 137.

REYNOLDS J., GYURE L., ANDREW E. & HALL J.G. (1980) Studies on the transport of polyclonal IgA antibody from blood to bile in rats. *Immunology*, **42,** 83.

YOFFEY J.M. & COURTICE F.C. (1970) Lymph flow from regional lymphatics. In: *Lymphatics, lymph and the lymphomyeloid complex*, p. 206. Academic Press, London.