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REPOPULATION WITH IgA-CONTAINING CELLS OF BRONCHIAL AND INTESTINAL LAMINA PROPRIA AFTER TRANSFER OF HOMOLOGOUS PEYER'S PATCH AND BRONCHIAL LYMPHOCYTES[1, 2]

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Transfer of 50 million rabbit allogeneic lymphocytes from either bronchus-associated lymphoid tissue (BALT) or Peyer's patches into 1000 R x-irradiated recipients results, 6 days later, in predominant repopulation of gut and bronchial lamina propria, as well as spleen, with IgA-containing cells.

After repopulation with BALT or Peyer's patch cells, lymphoid follicles in both gut and lung showed peripheral cellular membrane type of fluorescence with fluorescein-conjugated anti-IgA antisera only.

Six days after x-irradiation alone, little evidence of repopulation was seen and immunofluorescent qualitative observations of gut and lung, and quantitative data in the spleen, confirmed these findings. After transfer of 50 million lymph node cells, very few immunoglobulin-containing cells were seen in the gut or bronchial lamina propria.

These results suggest that there may be a common mucosal immunologic system, and that repopulation of gut and lung lamina propria may be through the organized lymphoid tissue therein.

We have recently described the presence in bronchial tissue of subepithelial lymphoid aggregates with remarkable morphologic similarity to Peyer's patches (1, 2). This bronchus-associated lymphoid tissue (BALT)[4] possesses a lymphoepithelium and does not stain with immunofluorescent reagents directed against IgG, IgA, or IgM. BALT is present in numerous mammals including man, as well as in chickens (1). The BALT is distributed at random along the bronchial tract and is also concentrated around bifurcations.

We wished to find out whether BALT contained a population of precursor cells destined to synthesize primarily IgA, as has been shown by Craig and Cebra (3) for Peyer's patches. The current report presents evidence that in this sense BALT and Peyer's patches share a similar function. Moreover, 6 days after transfer of either BALT or Peyer's patch cells into lethally irradiated rabbits, repopulation of both gut and bronchial lamina propria occurred predominantly with IgA-containing cells.

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[2] Presented in part at the American Federation of Biological Societies' 58th Annual Meeting, Atlantic City, 1974.

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[4] Abbreviations used in this paper: BALT, bronchus-associated lymphoid tissue; RPMI, Roswell Park Memorial Institute; HBSS, Hanks' balanced salt solution; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

MATERIALS AND METHODS

Animals. Seven-week-old female New Zealand white rabbits, average weight 2 kg were obtained from the Canadian Breeding Laboratories, Montreal, P.Q., and received food and water *ad libitum*. All animals received sulfaquinoxaline in their drinking water for 2 weeks before the experiment. All recipient rabbits were injected intramuscularly daily with gentamicin (2 mg/kg body weight) starting 1 day before irradiation and continuing until the animals were sacrificed.

Cell suspensions. The distal ileal Peyer's patch was excised from a rabbit anesthetized with pentobarbital. The mucosa was trimmed and the patch rinsed in saline containing 50 μg gentamicin/ml. A cell suspension was made in a homogenizer with a loose fitting plunger in RPMI 1640 containing 15% heat-inactivated normal rabbit serum (from a large pool of rabbit serum), 10 units of heparin, and 50 μg gentamicin/ml (RPMI). Cell clumps were allowed to settle for a few minutes at room temp and the supernatant was centrifuged at room temp for 5 min at 300 × G. The pellet was resuspended in RPMI, cells were counted in a hemocytometer, and adjusted to a final concentration of 50 × 10^6 cells/5 ml of medium. The cells were kept at 4°C until used for cell transfer. Cell viability was always greater than 90% as established by the trypan blue exclusion technique and the cell population consisted of greater than 90% lymphocytes.

Lymph node cells were obtained from both popliteal lymph nodes which were minced finely with scissors, homogenized, and then handled exactly as described for Peyer's patches.

BALT cells were prepared from several rabbits killed with an overdose of pentobarbital. The mucosal membrane was stripped from the lung parenchyma, washed in Hanks' balanced salt solution with 5% bovine serum albumin (HBSS-BSA) and ground in a loose fitting teflon homogenizer. Clumps and tissue debris were allowed to settle briefly at room temperature and the dissociated cells were obtained from the supernatant by centrifugation at 300 × G for 5 min and washed once in HBSS-BSA. The cells were then placed on a discontinuous BSA gradient (4) and centrifuged. The interface between 10 and 28% was removed, washed, and resuspended in HBSS-BSA. Better than 90% of these cells were lymphocytes with a viability greater than 95% according to the trypan blue exclusion technique. In order to obtain the 50 million cells necessary for the transfer experiments, BALT cells from individual rabbits were prepared separately and pooled to give a final concentration of 50 × 10^6/5 ml of medium containing 10 units/ml heparin. Less than 1% of these cells stained for cytoplasmic immunoglobulin with specific anti-heavy chain-conjugated reagents (see below). The first two BALT cell transfer experiments in Table I utilized cells prepared in this manner.

A second preparative technique was used for the remainder of the BALT transfer experiments, since it was found to be more reproducible and to give a higher yield. The tissue was taken as far as the homogenization step and clumps were allowed to settle at room temperature as for the first method. The supernatants were then centrifuged at 300 × G for 10 min at 4°C in siliconized glass tubes. The pellet was resuspended in low ionic strength buffer according to the methods of von Boehmer and Shortman (5). The cells were then applied to a 3-mm high cotton wool column made from a Pasteur pipette, and eluted with RPMI at 4°C. The dead cells clumped and were trapped in the column. The column effluent was spun at 300 × G for 10 min in siliconized tubes, washed twice, and resuspended in 5 ml RPMI and heparin (10 units/ml) at a final concentration of 10×10^6/ml.

With this method, the average yield per donor animal was 8×10^8 cells of which more than 90% were lymphocytes with greater than 98% viability. Cells from individual donors were injected i.v. separately until a total of 50×10^6 viable lymphoid cells per recipient had been reached.

Rabbits were irradiated singly under pentobarbital anesthesia on a turntable (8 rpm) exposed to a vertical beam of x-rays delivered by a General Electric Maxitron 250 therapy unit. The conditions of irradiation were: 250 kvp, 30 ma, 0.5 mm copper and 1.0 mm aluminum filters, half value layer 1.43 mm copper. The tissue dose for each animal was 1000 R (Total body) measured at mid-plane, 80 cm from centre line and the rate was 54.1 R/min.

Cell transfer. Each recipient received 50×10^6 cells via the marginal ear vein at a concentration of 10×10^6 cells/ml. These cells were injected slowly over several minutes within 3 hr of irradiation. Some animals died acutely within the 1st hr and several died within the first 24 hr. These problems were minimized with the BALT injections by keeping the cells from different animals separate.

Tissue processing. Six days after cell transfer or x-irradiation, rabbits were killed by neck fracture, and the spleen, intestine with a Peyer's patch, and bronchus taken around the bifurcation of a minor stem bronchus from the major bronchus, were removed. Two separate segments of spleen, intestine, and BALT were fixed in 10%-buffered formalin at 4°C for 4 hr (6), placed in 30% sucrose at 4°C for 18 hr, snap-frozen in methyl-butane cooled in liquid nitrogen, mounted in Cryocut and 4-μ sections were cut on cryostat. The sections were picked up on warm gelatinized slides, air dried, and then fixed in 10%-buffered formalin for 15 min. The sections were then washed for 20 min in phosphate-buffered saline, pH 7.4 (PBS), stained with Lendrum's stain to reduce nonspecific fluorescence (7) and stained with specific goat anti-rabbit heavy chain reagents prepared as previously described (1). The prior staining with Lendrum's did not interfere with fluorescence performed on normal rabbit tissues. The slides were then washed in PBS for 30 min and mounted in glycerol buffered with PBS. Appropriate controls were performed as reported elsewhere (8).

Slides were viewed in a Leitz fluorescent microscope equipped with Ploem incident light optics. Slides were occasionally viewed in transmitted light with an HB 200 mercury lamp source with a KP 490 filter.

Photomicroscopy. Photographs of tissue sections and cells were taken on Kodak high speed daylight Ektachrome film, ASA 160. On average, exposures were 60 sec.

Counts of spleen cells with cytoplasmic fluorescence. At the time of sacrifice 6 days after the cell transfer, a segment of spleen was minced with fine scissors in HBSS-BSA and dispersed in a homogenizer fitted with a loose pestle. Clumps of debris were allowed to settle for a few minutes at room temperature and the supernatant was removed, centrifuged at 300 × G for 10 min and the pellet was resuspended in HBSS-BSA to give a final nucleated cell suspension of 2×10^6 cells/ml. Cell smears were prepared on a Shandon cytocentrifuge from 0.15 ml of each cell suspension centrifuged for 10 min at 126 × G. The cells were immediately fixed damp at room temperature by immersion in 95% ethanol for 30 min, washed in PBS, and stained with the appropriate immunofluorescent reagent. The number of cells staining with a given reagent was determined per 10,000 cells by counting consecutive fields, using alternate mercury and tungsten light sources per field, in order to determine the total numbers of cells in every field. The counting was performed at a constant magnification (400 ×).

RESULTS

Control studies

Quantitative determination

Normal spleen. As can be seen in Table I the mean number of IgG-containing cells, which predominated in normal spleen, amounted to $120/10^4$. The second most frequent immunoglobulin class was that of IgM numbering 99; and the third, IgA amounting to 55 cells per 10^4 spleen cells.

X-irradiation control spleen. To examine the effect of irradiation alone and the potential regeneration of cells at 6 days, or cells left unaffected by the irradiation, we examined splenic tissues from animals which had only received irradiation. The results can be seen in Table I. The number of positive cells in the spleen was reduced to about 10% of normal. The relative numbers of IgG-, IgM-, and IgA-containing cells compared to the total number of immunoglobulin-containing cells and number in each class were, however, retained.

Qualitative determination

Normal spleen. The visual impressions corresponded well to the quantitative results and were similar to those reported by Crandall et al. (9).

X-irradiation control spleen. The spleen was largely devoid of immunoglobulin-containing cells. Occasional randomly distributed cells positive for cytoplasmic fluorescence were seen. No evidence of repopulation of the spleen was found.

X-irradiation control intestine. Intestinal villi completely lacked immunoglobulin-containing cells of any class. Peyer's patches appeared to be necrotic with a major orange autofluorescence found partly dispersed throughout the follicles but also particularly prevalent in the centers. It is possible on normal Peyer's patches with potent anti-IgA and anti-IgM antisera to see membrane immunofluorescence of these cells especially in the centers of the Peyer's patch follicles. No such fluorescence was seen in these sections and there was no sign of repopulation.

X-irradiation control lung. The lymphoid areas were devoid of immunoglobulin-containing cells as they are in normal animals (1). The lymphoid tissue was in all cases completely destroyed and hard to distinguish in dark field immunofluorescence. However, no positive cells were found within the follicles, the bronchial lamina propria, or the alveolar tissues.

TABLE I

Number of immunoglobulin containing cells per 10^4 spleen cells 6 days after cell transfer of 50 × 10^6 lymphocytes into 1000 R irradiated recipients

Donor Cell Source	Animal	Immunoglobulin		
		IgA	IgG	IgM
X-irradiation control	1	0	18	10
	2	0	27	19
	3	12	9	10
	4	7	6	7
Mean ± S.E.[a]		5 ± 3	15 ± 5	12 ± 3
Normal Spleen[b]	1	35	113	105
	2	74	127	83
Mean		55	120	99
BALT	1	119	13	32
	2	781	17	19
	3	562	59	32
	4	267	32	34
	5	1148	128	146
Mean ± S.E.		575 ± 184	50 ± 24	53 ± 26
Peyer's patch	1	1690	30	46
	2	180	56	52
	3	464	53	71
	4	1148	87	70
	5	534	53	59
Mean ± S.E.		803 ± 203	56 ± 10	60 ± 6
Popliteal node	1	210	275	245
	2	176	384	62
Mean		193	329	154

[a] S.E., standard error.
[b] Number of cells containing immunoglobulin in a normal spleen.

Peyer's patch cell transfer

Quantitative

As previously shown by Craig and Cebra (3) in this model the spleen possessed large numbers of cells containing IgA with a mean for five such experiments of 803 relative to 56 IgG- and 60 IgM-containing cells (Table I).

Qualitative

Spleen. Colonies of IgA-containing cells, varying in size and always surrounding central blood vessels were seen. The scanning of tissue sections correspond extremely well to the numbers obtained on cell suspensions. Large colonies were seen with central areas of cells lacking intracellular IgA but with peripheral staining for immunoglobulin. Smaller colonies with IgM cytoplasmic fluorescence were also seen. IgG-containing cells were scarce although a few groups of such colonies were found.

Intestine. Villi were repopulated with IgA containing cells which were often more numerous toward the bases. IgA-containing cells were especially numerous in areas immediately adjacent to the Peyer's patches themselves. In tissue sections from animals which appeared to be more highly repopulated, both in quantitative counts and also on tissue sections of spleen, repopulated areas were seen within Peyer's patches with cells with peripheral staining for IgA (Fig. 1). Cells were rarely found within Peyer's patches with cytoplasmic fluorescence with any heavy chain-specific reagents. Very few IgG- or IgM-containing cells were apparent in the villi in any of the sections scanned either in the lamina propria or adjacent to the Peyer's patches themselves.

Lungs. A cluster of subepithelial IgA-containing cells can be seen in Figure 2 in an area adjacent to a BALT follicle. This was often found with Peyer's patch cell transfers. The numbers of such cells were always limited. Occasional single cells were found in the lung parenchyma unrelated to lymphoid areas. Peripheral staining of lymphoid cells was occasionally seen in the follicles themselves very similar to that described above for Peyer's patches. IgG- and IgM-containing cells were not found in BALT sections after transfer of Peyer's patch cells.

BALT cell transfer

Quantitative

Six days after the transfer the spleen possessed a mean of $575/10^4$ IgA-containing cells as seen in Table I. This figure was 10 times the number of cells seen with IgG or IgM.

Qualitative

Spleen. Colonies of IgA-containing cells were generally seen around central blood vessels (Fig. 3). Smaller colonies of IgM- and IgG-containing cells were also found similar to the results obtained with Peyer's patch cell transfers. Individual distinct cells with cytoplasmic staining for IgA, IgG, or IgM were found

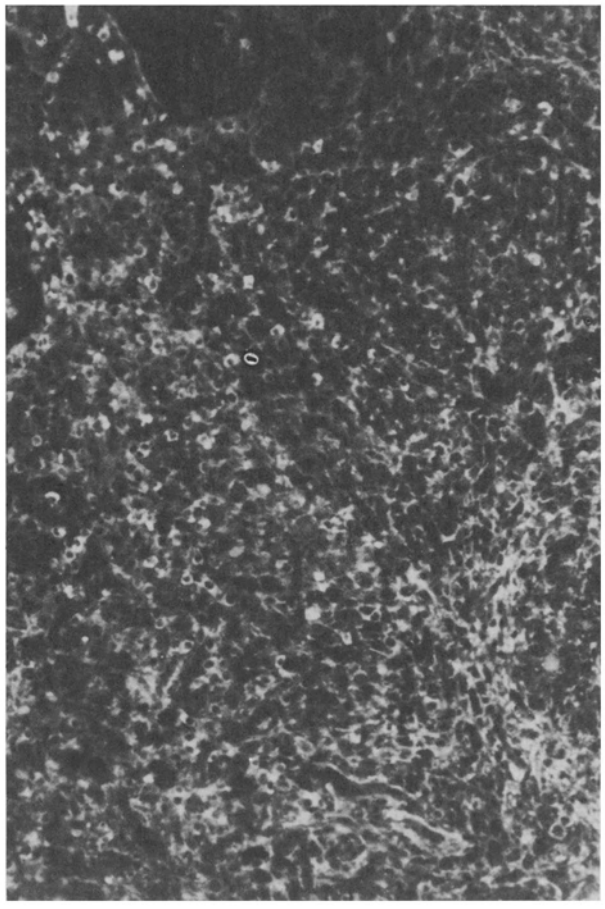

Figure 1. Detail of Peyer's patch after Peyer's patch cell transfer. Note peripheral membrane fluorescence with anti-IgA antiserum (× 250).

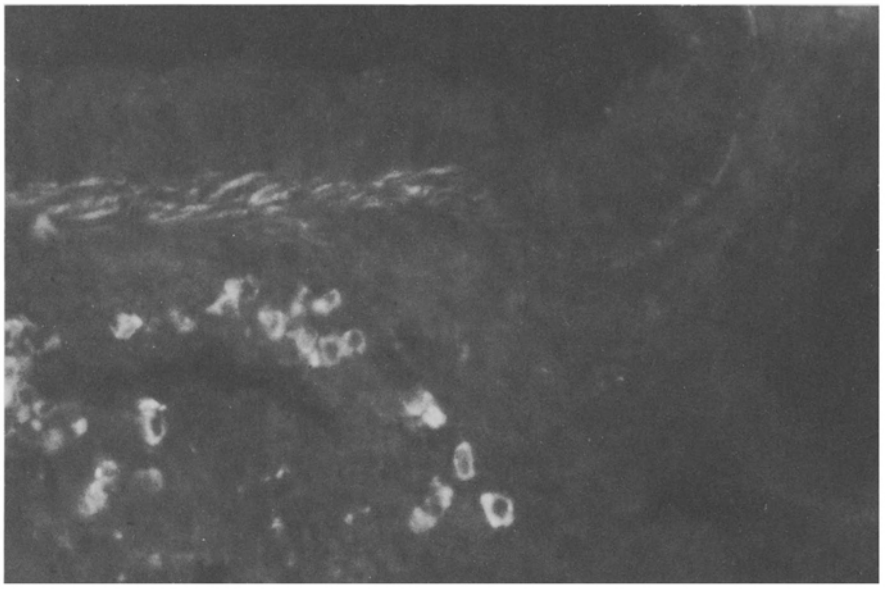

Figure 2. BALT with few IgA-containing cells at periphery of follicle after BALT cell transfer. Cartilage at right, epithelium at top of picture (× 400).

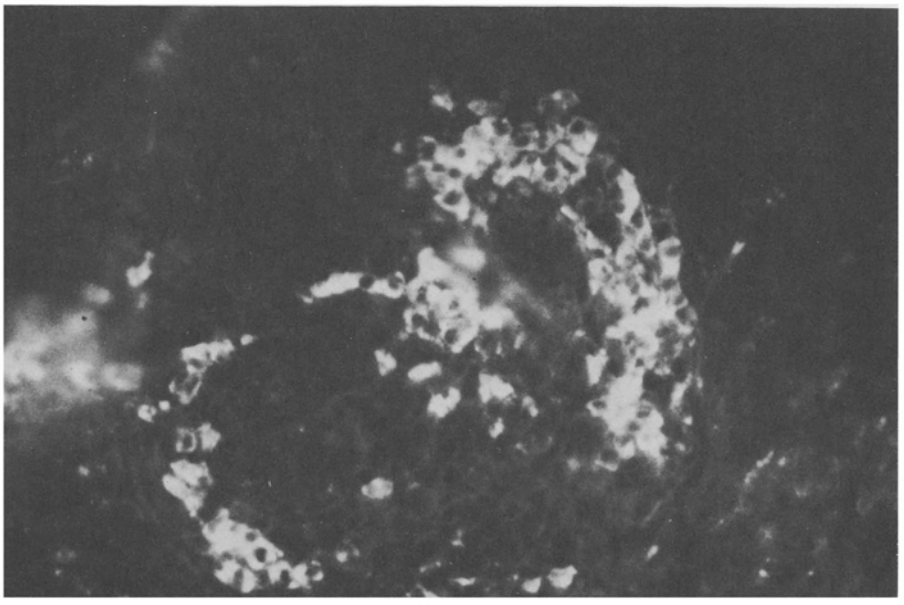

Figure 3. IgA-containing cells in spleen after BALT cell transfer (× 400).

apparently randomly scattered throughout the splenic tissue.

Intestine. As with the tissues of recipients of Peyer's patches IgA-containing cells were found throughout the villi and again were on the whole more numerous toward the bases of villi and especially numerous around Peyer's patch follicles. Only very rare cells staining positively for IgG or IgM were found (Fig. 4). Repopulation of follicles with cells possessing membrane-positive IgA was occasionally seen.

Lung. Individual separate IgA-containing cells were seen randomly scattered in lung parenchyma. Small groups of IgA-containing cells were also seen in the bronchial lamina propria and adjacent or close to areas of BALT, for example between cartilage plates. BALT follicles were occasionally seen in which the centers of such follicles appeared to be repopulated with cells whose membranes stained positively for IgA. No IgM or IgG cells were found after repopulation with BALT in the bronchial lamina propria or lung tissue.

Popliteal lymph node

Quantitative

After popliteal lymph node cell transfer into irradiated recipients IgG-containing cells predominated in the spleen. These cells were approximately twice the number of IgM- or IgA-containing cells.

Qualitative

Spleen. The findings correspond well to the quantitative results. The colonies consisted of a few to large numbers of cells clustered around central blood vessels. Little difference in distribution was seen in this respect between the immunoglobulin classes.

Intestine. No evidence of repopulation of villi was found after popliteal lymph node transfer. Very occasional cells staining with IgG were seen. Cells staining with IgM were not found and in the sections viewed no cell staining for IgA was perceived. No evidence of repopulation was found in or adjacent to the Peyer's patches.

Lung. Very rare cells containing IgG were found apparently randomly scattered in the bronchial lamina propria. No cell staining for IgA or IgM was found in any of the sections viewed. No evidence of repopulation adjacent to or within the BALT follicles was found.

DISCUSSION

BALT possesses many characteristics similar to the aggregated mucosal lymphoid tissue of the gut. BALT is found in the

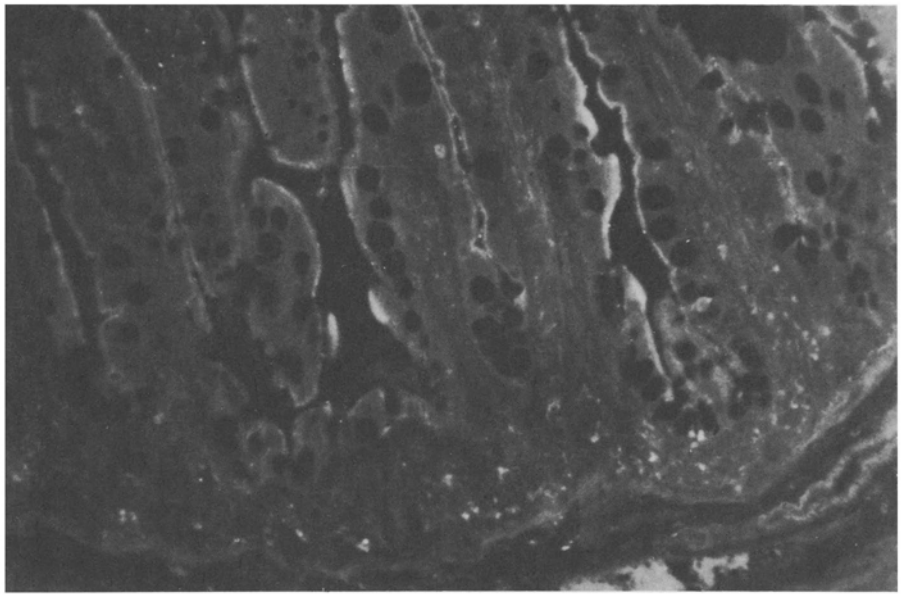

Figure 4. Intestine stained for IgG-containing cells after BALT cell transfer. Very little cellular fluorescence (× 100).

bronchial tract, particularly at bifurcations (1, 2). It possesses follicles with an overlying lymphoepithelium which is heavily infiltrated with lymphocytes and is flattened and usually devoid of cilia and glands. The follicles are not stained with fluorescein conjugated anti-heavy chain reagents. On the basis of *in vivo* labeling experiments with ^{3}H-thymidine (10), and also autoradiography of tissue sections after *in vivo* infusion of ^{3}H-thymidine- or ^{3}H-uridine-labeled thoracic duct cells (10), the BALT lymphocytes appear to consist of cells partly derived locally and partly from the circulating pool. Recent experiments have shown that, similar to Peyer's patches, a predominant proportion of BALT lymphocytes possess α or μ heavy chain membrane markers (11).

Because of these data we wished to compare the results of infusion of Peyer's patch cells with those obtained after BALT transfer, using the 1000 R irradiated rabbit model already explored by Craig and Cebra (3). We have, in this series of cell transfer experiments, confirmed these authors' results that Peyer's patches possess a population of precursor cells destined for IgA synthesis. We have further shown that the BALT also contains a precursor population for IgA synthesis, similar to Peyer's patches. Our repopulation studies strongly support the concept (1) that the subepithelial lymphoid aggregates of the lung and gut are part of a common mucosal immunologic system.

Craig and Cebra have shown, using allotype markers, that the majority of cells synthesizing immunoglobulin after allogeneic cell transfer in this model are of donor origin (3). The differences in our experiments between the animals that received x-irradiation alone, and the control animals, was such that we feel that it is reasonable to assume on the basis of the evidence quoted that our results also reflect primarily donor cell synthesis.

Repopulation of spleen by donor cells synthesizing IgA may not necessarily be a true indication of such cells' potential. We have carried out similar transfer studies using autologous Peyer's patch cells (12) and have shown that few IgA-staining cells are found under these conditions in the spleen. However, the predominant repopulation of both gut and lung lamina propria after autologous Peyer's patch cell transfer was by IgA-containing cells. We have postulated (12) that the splenic IgA synthesis found in the homologous transfer experiments used in the present paper may be due to an allogeneic effect, whereas the synthesis of IgA in the musoca may be antigen driven.

With the homologous system both BALT and Peyer's patch cell transfer resulted in predominantly IgA synthesis in the spleen, whereas with lymph node cells the IgG class predominated. Regardless of the mucosal cell source essentially only IgA production was seen in both gut and lung. Lymph node cell transfer resulted in very rare immunoglobulin containing cells of any class in the mucosal lamina propria of the gut or lung.

After transfer, Peyer's patch cells tended to repopulate areas closer to Peyer's patches. Immunofluorescent studies of the distribution of IgA-containing cells in normal gut have shown that these cells are more frequently found adjacent to the patches (13). Our findings of peripheral, maybe membrane staining of cells in some but not all Peyer's patches as well as some BALT follicles might tend to support the argument that cells going on to IgA synthesis may first pass through the organized mucosal lymphoid tissue. Experiments with mouse Peyer's patch cells labeled with ^{51}chromium have shown that only a portion of these cells return to the patches, much less go to the lamina propria, and the majority go to the lymph nodes and spleen (14). These studies, however, have been performed as homing experiments, so that data are only available, at most, up to 48 hr after transfer. It is quite possible that initial homing and final site may not be the same, or that our results may only reflect a relatively small population of cells contained in the original Peyer's patch or BALT population actually destined to go on to IgA production.

The concept of local mucosal immunity (15) has so far stood the test of time. All evidence points to the fact that in man as well as in animals, in order to initiate a local immune response the antigen must be delivered to the mucosal site, either by oral feeding in the case of the gut, or by a parenteral injection of a sufficiently large dose of antigen to allow it to reach the lamina propria through the systemic circulation (16).

How can the results reported here be explained since they appear to contradict the concept of local mucosal immunity? Only a few IgA-containing cells are found in the normal bronchial lamina propria (17). The lung is relatively protected in so far as antigenic stimulation is concerned, and certainly so when compared to the gut. The BALT follicles are small and may be present in tissue maintained in germ-free and apparently antigen-free environments (2, 18). However, on immunization, the bronchial lamina propria can respond, depending upon the antigen, with a massive local IgA-containing cell

response. Such respiratory responses have been seen with Sendai virus in mice (19) and cystic fibrosis in man (20). Further, the absence of antibody in secretions of the oropharynx after colonic immunization does not exclude the possibility that cells were present in the mucosa of the oropharynx committed to specific IgA antibody production (21). The sensitivity of the techniques used to detect viral antibody in these secretions ranges normally up to titers of 1:32. The facts that injected BALT cells migrate to the gut and that quantitatively almost as many IgA cells are found after BALT transfer in the spleen as with Peyer's patch transfer, strongly suggest a common function of those antibody cell precursors. This explanation may also account for the observation that military recruits have apparently been almost totally protected against infection by adenovirus type 4 administered orally in enteric coated capsules, despite the absence of nasal secretion antibody and limited serum antibody (22, 23).

Both bronchial and gut mucosal surfaces are characterized by IgA. Both are derived from endoderm, and both possess mucosal lymphoid aggregates with many morphologic and functional characteristics in common. Both organs have lymphoid aggregates at sentinel sites guarding the portals of entry of potential pathogens.

Ham (24) has suggested that mucosal lymphoid follicles are a characteristic of wet epithelial surfaces. A system of immunity initiated locally but also geared to send out precursors of antibody producing, as well as perhaps sensitized T cells, to other mucosae would be an extremely efficient immunologic system. We believe that a mucosal immunologic system common to several mucosal surfaces may exist. The observation that feeding of antigen to rabbits can result in high levels of secretory IgA antibody in colostrum would support this view (25).

The implications of our findings open to testing are that oral immunization may prove to be effective in the protection of several mucosal surfaces.

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