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Germinal Centres and the Origin of the B-cell System

I. GERMINAL CENTRES IN THE RABBIT APPENDIX

P. NIEUWENHUIS, C. E. VAN NOUHUIJS, J. H. EGGENS AND F. J. KEUNING

Department of Histology, University of Groningen, Oostersingel 69[1], *Groningen, The Netherlands*

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Summary. Regeneration of follicular structures in the rabbit spleen after a single dose of 450 rads whole body X-irradiation was shown to be dependent upon the presence of the appendix.

Results obtained were in favour of a stream of cells—probably derived from appendix germinal centres—to follicular structures in the spleen, in particular to the lymphocyte corona and marginal zone but excluding germinal centres.

Recovery of the antibody forming potential following X-irradiation was equally shown to be dependent upon the presence of the appendix.

It was concluded that following 450 rads whole body X-irradiation the appendix contributes to the recovery of the antibody forming capacity of the spleen by the production of antibody-forming cell precursors; these latter cells are presumably identical with the follicle replenishing class of lymphocytes derived from appendix germinal centres.

INTRODUCTION

The B-cell system is often described in negative terms as that part of the lymphoid system which is non-thymus-derived as to its origin, and non-thymus-dependent as regards its development. However, sometimes it may be thymus-dependent in its function.

In mammals and birds its function is related to antibody formation. It is believed to include all antibody-forming cell precursors which upon appropriate stimulation by antigen transform into antibody-forming and secreting cells of the plasma cell series.

In mammals B cells have been demonstrated to be ultimately bone marrow-derived (Miller and Mitchell, 1969). In birds the B-cell system is supposed to be derived from the bursa of Fabricius (Cooper, Peterson and Good, 1965). By analogy, in mammals a bursa equivalent has been suggested as an essential 'staging-post' in the development of the B-cell system (Cooper, Perey, McKneally, Gabrielsen, Sutherland and Good, 1966; Good, Cain, Perey, Dent, Meuwissen, Rodney and Cooper, 1969).

From a morphological point of view definition of the B-cell system again depends on the knowledge of the T-cell system; it is that part of the lymphoid system which is not depleted by either neonatal thymectomy alone or adult thymectomy followed by lethal X-irradiation and bone marrow reconstitution. In general follicular structures (primary nodules and secondary nodules) are thought to represent morphologically a major part of the B-cell system.

In the present series of papers an attempt is made to correlate histological observations on the B-cell system with its known functional aspects and to describe its cellular kinetics in both morphological and functional terms.

In initial experiments (Keuning, Bos, van den Broek and Veldman, in preparation) regeneration of follicular structures after X-irradiation (either locally to popliteal lymph nodes or whole body) in the presence or absence of the thymus was investigated. It was shown that in peripheral blood there existed a thymus-independent population of lymphocytes which was capable of replenishing follicles. The presence or absence of this population was correlated with the capacity to give a primary antibody response upon proper antigenic stimulation.

In the present paper the relation between the appendix (as a postulated bursa-equivalent) and this follicle-replenishing class of lymphocytes is investigated.

MATERIALS AND METHODS

Animals

Young adult 4–5-month-old rabbits (Gold–Agouti) were obtained from the Centraal Proefdierenbedrijf/TNO, Zeist.

Antigens

0·2 ml of a suspension of formol-killed *Salmonella java* paratyphi B (rich in H-antigen) in saline (5×10^9 organisms/ml) was injected i.v. on the 10th day after irradiation (in the experiments indicated).

Antibody titration

Serum anti-H-agglutinin titres were determined using a Ficker suspension (R.I.V. Utrecht) as the test antigen. Doubling dilutions were made and the endpoint was taken as the highest dilution showing visible agglutination.

Light microscopy

Tissues were fixed in a mixture of Zenker's fluid, formalin 40 per cent and trichloracetic acid 2 per cent (90:5:5) for 5 hours at room temperature. After washing for 24 hours in running tap water, tissues were processed through graded alcohols, methyl benzoate (+1 per cent celloidin added) and benzene to paraffin wax. Sections were cut at 5–7 μm and stained with Methyl Green–Pyronin (Brachet, 1953).

Autoradiography

[^{3}H]thymidine (specific activity 5000 mCi/mM) was obtained from the Radiochemical Centre (Amersham). Tissues (slices approximately 1·5–2·0 mm thick) were fixed in 2 per cent glutaraldehyde in phosphate buffer (pH 7·4, milliosmolar $\pm$400) for 6 hours at room temperature. Tissues were washed overnight in 0·1 molar phosphate buffer with 6·8 per cent (w/v) sucrose added. After paraffin-embedding sections were cut at 5 μm and processed for autoradiography using the stripping film technique (Kodak AR-10) according to Pelc (1956). Following exposure for 3–6 weeks at 4° slides were developed using Kodak D19^B developer (10 minutes, 18°C), fixed, washed and stained through the film with Methyl Green–Pyronin.

X-irradiation

A Philips–Mueller (MG 300) X-ray machine was used as the radiation source (kindly provided by Professor Dr Lamberts, Department of Radiopathology, Groningen). The machine was operated at 200 kV and 15 mA; primary filters 0·5 mm Cu and 0·6 mm Al; H.V.L. 1·0 mm Cu. Dose rate at 80 cm 21 rad/minute. Back scatter was included in all dosages.

Whole body X-irradiation

Animals were subjected to whole body X-irradiation, half of the radiation dose being given from the left, the other half from the right, so as to achieve a homogeneous radiation dose of 450 rads throughout the body.

Whole body X-irradiation with the appendix shielded

One week prior to irradiation the appendix was surgically fixed in the midline to the inner side of the abdominal wall. During irradiation (see above) the appendix was shielded by a curved lead shield the length of the appendix placed under the abdomen rising on both sides for approximately 3 cm; when animals were irradiated from either left or right side this shield only protected the appendix fixed in the midline.

Surgical procedures

All surgical procedures were performed under aseptic conditions. Animals were anaesthetized with 0·7–1·0 ml pentobarbital (60 mg/ml) i.v. and 0·25 ml Vetranquil i.m.

Appendectomy

The appendix was exteriorized through a midline incision in the ventral abdominal wall. Branches from the appendicular artery to the small intestine were ligated and cut. At the base of the appendix the appendicular artery and vein were ligated and cut. The appendico–caecal junction was severed between two ligatures and the distal end of the caecum was invaginated with a purse-string suture and covered with peritoneum. Globenicol powder was sprayed in the operation area, after which the wound was closed.

Appendicostomy

After mobilizing the appendix as described above the appendico–caecal junction was ligated but not cut. Care was taken to leave the vasculature of the appendix intact. The distal end of the appendix was then exteriorized and during closure of the wound was sewn into the muscle layer of the abdominal wall, its tip protruding for approximately 1 cm. After cutting off the tip of the appendix the remainder of the appendicular wall was then sewn to the surrounding skin by an everting lockstitch suture using atraumatic silk sutures. To remove the appendiceal contents the appendiceal lumen was washed several times with sterile saline and finally approximately 5 ml of a mixture of neomycin/phthalylsuphlathiazole in saline (1:1:100, w/w/v) was instilled into the lumen of the appendix. Washings with this 'sterilizing' mixture were performed on alternate days till the end of the experiment.

Labelling procedures

Local [^{3}H]thymidine labelling of the appendix was achieved in either of two ways.

(1) *By arterial perfusion.* A PVC catheter (o.d. 0·625 mm, i.d. 0·5 mm) fitted with a gauge

21 needle was used to cannulate in a retrogade direction the most proximal branch of the appendicular artery supplying the small intestine. Through this branch 100 μCi of [^{3}H]thymidine in 1 ml of saline was delivered into the appendicular artery in ten short pulses (5 seconds each, one pulse per minute) of 0·1 ml by means of a perfusion pump fited with a 2-ml syringe.

To check perfusion efficiency Trypan Blue was added to the perfusion fluid. With this method fairly homogeneous labelling of the distal three quarters of the appendix was achieved. Control biopsies of the spleen taken 1 hour after perfusion of the appendix did not show labelling.

(2) *By a single i.v. injection of* [^{3}*H*]*thymidine.* 0·2 μCi/gram of body weight was used 18 hours after 450 rads whole body X-irradiation with the appendix shielded (Süssdorf, 1960). As shown by Nygaard and Potter (1959) 18 hours after 450 rads whole body X-irradiation uptake of [^{3}H]thymidine in irradiated tissues is markedly reduced. Thus most of the labelling was confined to the shielded appendix. In control biopsies of the spleen taken 1 hour after labelling only very few lightly labelled cells were detected, mostly plasma cells.

RESULTS

POST-IRRADIATION REGENERATION OF FOLLICULAR STRUCTURES IN THE SPLEEN

Animals were subjected to 450 rads whole body X-irradiation (1) in the absence of the appendix following appendectomy 1 week prior to irradiation, (2) with the appendix shielded during the irradiation, or (3) unmanipulated (controls). Biopsies of the spleen were taken 1, 2, 4, 7, 10 or 14 days after the irradiation. Regeneration of follicular structures in the spleen was expressed in a semi-quantitative way as +, ++, +++ and ++++. Results are shown in Fig. 1.

In control animals regeneration of follicular structures was witnessed by the 7th day after the irradiation by the appearance of small groups of lightly pyroninophilic, medium-

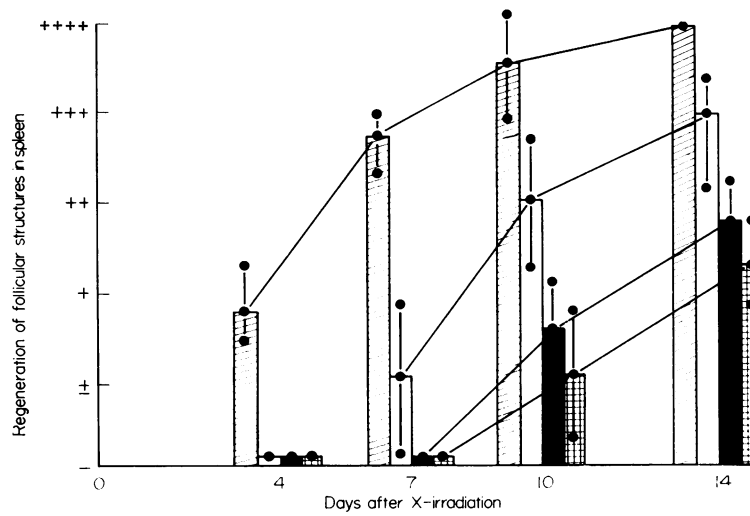

FIG. 1. Regeneration of follicular structures in the spleen after 450 rads whole body X-irradiation: (▨) with the appendix shielded during the irradiation; (□) controls, (■) following appendectomy prior to the irradiation and (▦) following appendicostomy and sterilization of the appendiceal lumen. Mean and range are indicated for each group.

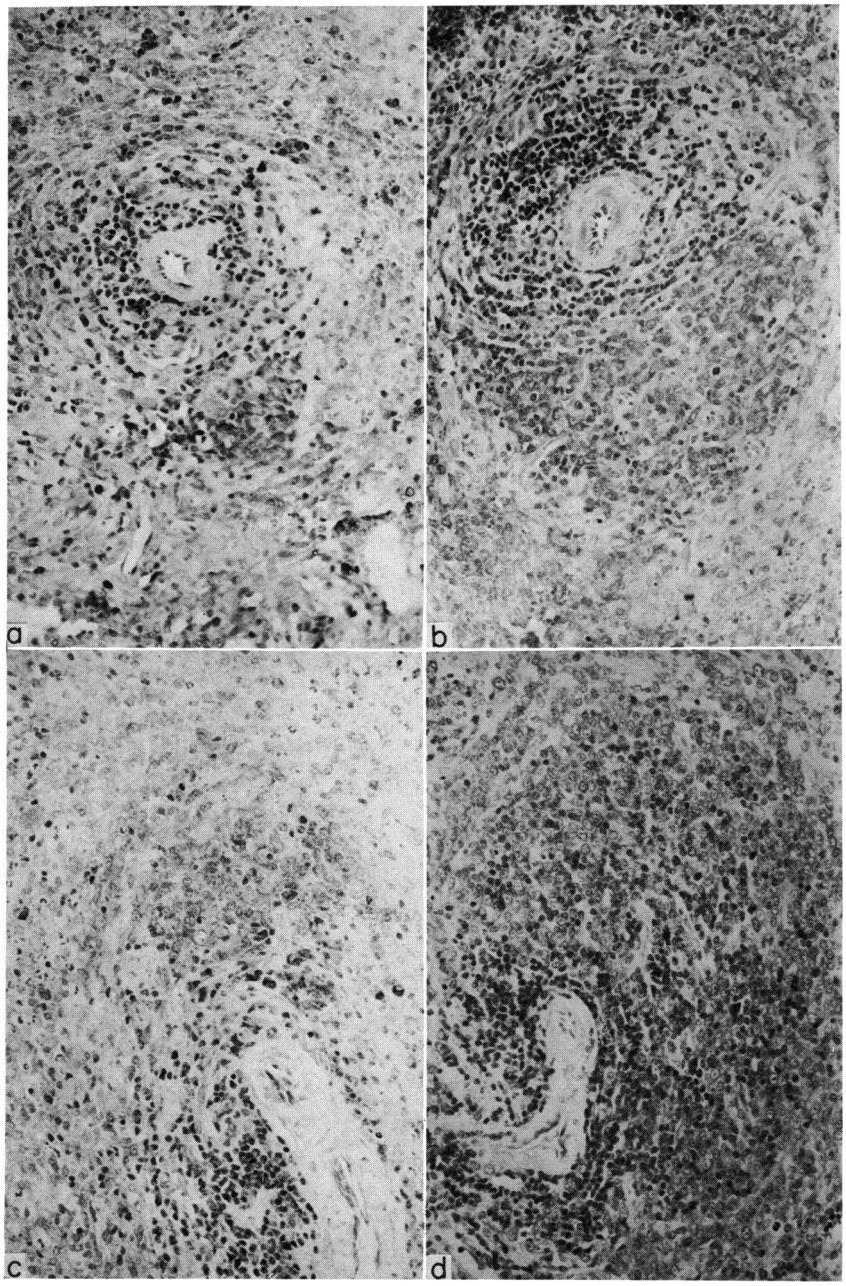

FIG. 2. Regeneration of follicular structure in the spleen after 450 rads whole body X-irradiation: (a) 7 days after X-irradiation (control); (b) 10 days after X-irradiation (control); (c) 10 days after irradiation of previously appendectomized animal; (d) 7 days after irradiation with the appendix shielded. Note (c) deficient regeneration in the absence of the appendix and (d) enhanced regeneration when the appendix was shielded during the irradiation. (Magnification × 150.)

sized lymphoid cells resembling the marginal zone cells of secondary nodules. Between the 7th and 14th post-irradiation day the number of cells per regenerating follicle as well as the number of follicles was increasing rapidly. However, mitotic figures were only occasionally seen amongst these cells (Fig. 2a, b).

In contrast in the appendectomized and irradiated animals regeneration of follicular structures was delayed for about 3–4 days as compared to controls. The regeneration pattern, however, seemed to run a parallel course (Fig. 2c).

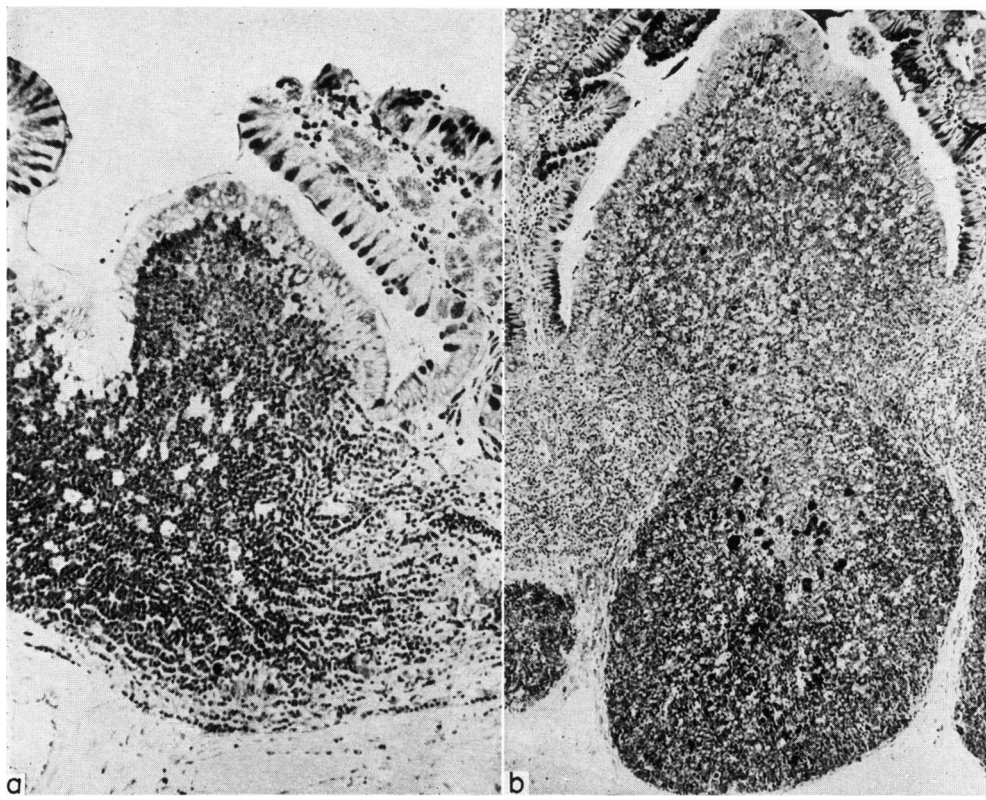

FIG. 3. Regeneration of follicular structures in the appendix following 450 rads whole body X-irradiation. (a) Four weeks after whole body X-irradiation following appendicostomy and sterilization of the appendiceal lumen. Note absence of germinal centres in wall of 'sterilized' appendix. (Magnification ×84.) (b) Ten days after whole body X-irradiation (control). (Magnification × 120.)

In the appendix-shielded and irradiated group early follicular regeneration was clearly distinguishable as early as 4 days after the irradiation. The appearance was restored to normal by the 10th or 14th day (Fig. 2d).

From these data it was concluded that the appendix contributes to the post-irradiation regeneration of follicular structures in the spleen. The simplest explanation of this contribution is a traffic of cells generated in the appendix to these structures in the spleen.

SOURCE IN THE APPENDIX OF CELLS CONTRIBUTING TO FOLLICULAR REGENERATION IN THE SPLEEN

Animals were subjected to appendicostomy and intraluminal 'sterilization' of the appendix 1 week prior to a single dose of 450 rads whole body X-irradiation. Biopsies of the spleen were taken 7, 10 or 14 days after the irradiation. Results are shown in Fig. 1. Although the appendix was present in these animals follicular regeneration in the spleen was delayed as in the appendectomized animals. Histological examination of the appendix of the appendicostomized and irradiated animals disclosed complete absence of germinal centres in contrast to the appendices of control animals (see previous section) where highly active germinal centres were found as early as 7 days after the irradiation (Fig. 3a, b).

It was concluded that the appendix-derived cell population contributing to follicular regeneration in the spleen was derived from the germinal centre compartment of (regenerating) follicular structures in the appendix.

EVIDENCE OF CELL TRAFFIC FROM THE APPENDIX TO FOLLICULAR STRUCTURES IN THE SPLEEN BY MEANS OF LOCAL LABELLING OF THE APPENDIX

Local labelling of the appendix was achieved (1) by arterial perfusion of the appendix or (2) by the i.v. injection of [^{3}H]thymidine 18 hours after whole body X-irradiation with the appendix shielded. Spleen biopsies were taken either (group 1) 48 and 72 hours after appendix perfusion, or (group 2) 4 and 7 days after the irradiation (approximately 3 and 6 days after labelling). In a supplementary series of experiments appendix biopsies were taken 1, 24, 48 or 72 hours after perfusion of the appendix (group 1).

Group 1

One hour after perfusion labelled cells were detected in the appendix: (a) in the subepithelial zone (dome-shaped area) about 5 per cent of the cells were labelled; (b) in the lymphocyte corona there was an occasional labelled medium-sized lymphoid cell; no label was detected over the small dark-staining lymphocytes making the bulk of the lymphocyte corona; (c) in the germinal centre label was largely restricted to the large pyroninophilic cells in the outer generative zone, or densely populated area (DPA); only an occasional medium sized lymphoid cell was found labelled in the more central part or thinly populated area (TPA). After 48 or 72 hours the most striking feature was the very substantial increase in the proportion of labelled cells in the subepithelial marginal zone (approximately 50 per cent) and the central part of the germinal centre (TPA) (approximately 100 per cent uniformly labelled) (Fig. 4). The number of labelled cells in the lymphocyte corona had not increased significantly.

In the spleen 48 or 72 hours after local labelling of the appendix, labelled small or medium-sized lymphoid cells were predominantly found in the lymphocyte corona and marginal zone of follicular structures (54 per cent); if the number of labelled cells found in the marginal zone surrounding the periarteriolar lymphocyte sheath (PALS) is added to this figure, as many as 88 per cent of all labelled cells were detected in non-thymus-dependent (B) areas. Twelve per cent of all labelled cells were detected in the PALS. Only one labelled cell was found inside a germinal centre.

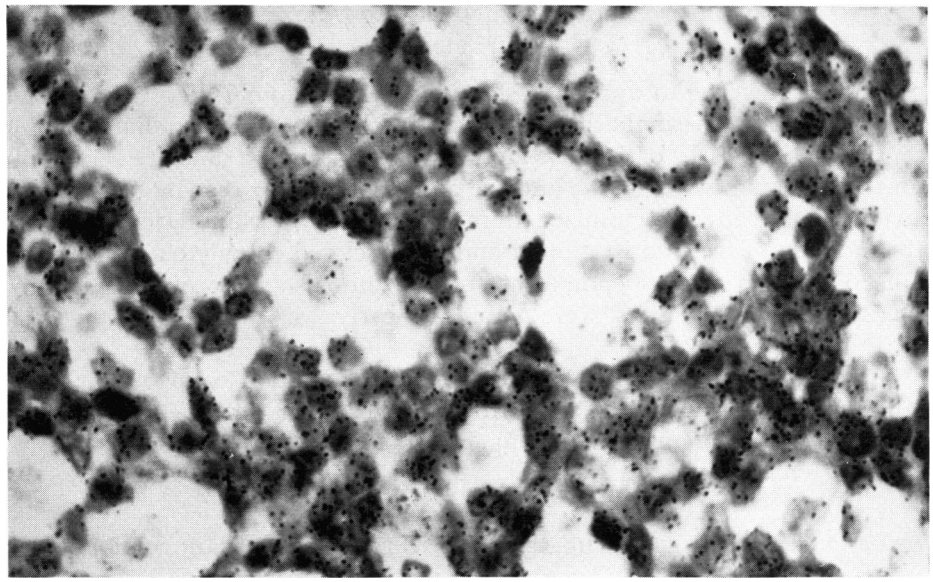

FIG. 4. Central part of appendix germinal centre, 3 days after local [^{3}H]thymidine labelling of the appendix. Note that virtually all small lymphocytes are labelled indicating production of these cells, presumably in the outer generative zone of the same germinal centre *in situ* (Magnification × 560.)

Group 2

Spleen biopsies taken 1 hour after the i.v. injection of [^{3}H]thymidine and 18 hours after the irradiation disclosed the normal picture of X-irradiation damage, i.e. complete absence of follicular structures and partial survival of the thymus-dependent population around the arterioles (PALS). In autoradiographs at this time only an occasional cell was found labelled. The incorporation pattern in the appendix was as described above after perfusion labelling, mean grain counts however being lower. Three days later spleen biopsies disclosed small groups of medium-sized lymphoid cells representing early follicular regeneration; by this time the majority of these cells were lightly labelled indicating their origin in the shielded and labelled appendix. By the 7th day after the irradiation follicular regeneration had progressed as described above; grain counts over these cells, however, were too low for definite conclusions to be drawn.

THE APPENDIX AS A SOURCE OF ANTIBODY-FORMING CELL PRECURSORS (AFCP'S)

To investigate the contribution of the appendix to the recovery of the primary antibody-forming potential following 450 rads whole body X-irradiation animals were challenged with a single dose of paratyphoid vaccine on the 10th day after the irradiation. In addition to this basic scheme some animals were appendectomized 1 week prior to the irradiation, a second group appendectomized immediately prior to the antigenic challenge and a third group splenectomized 1 hour after antigenic challenge. A fourth group was irradiated and challenged with the antigen and thus served as control.

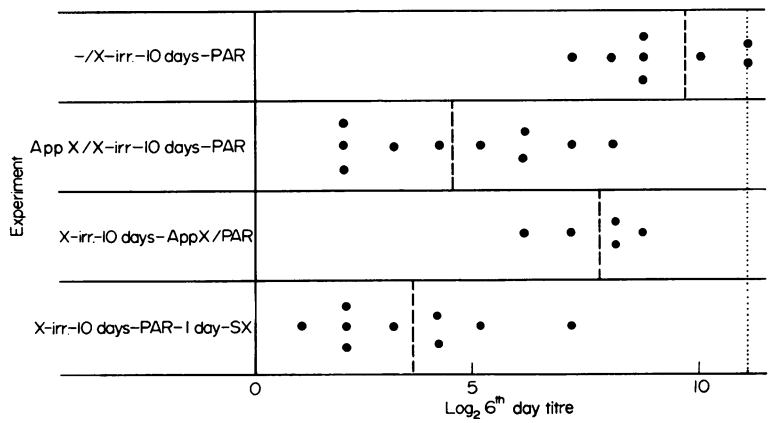

FIG. 5. The appendix as a source of antibody-forming cell precursors. The effect of appendectomy (App X) on the recovery of the primary antibody-forming potential (expressed as 6th day titre) following 450 rads whole body X-irradiation (X-irr.). Antigen (*Salmonella java* vaccine (PAR) was injected 10 days after X-irradiation. SX = splenectomy. (– – –) Indicates mean values for each group. (. . . .) Indicates control levels of 6th day antibody titre in antigen injected normal animals.

As can be seen from Fig. 5 in animals which had only been irradiated, antibody responsiveness—expressed as $\log_2$ 6th day titre—had returned to near normal by the 10th day after the irradiation. In contrast animals appendectomized prior to the irradiation showed a substantially lower antibody response. In animals appendectomized immediately prior to the antigenic challenge (10th day) serum antibody titres did not differ significantly from the non-appendectomized controls. This indicates that the appendix *per se* is not essential in the antibody response measured here. However, Fig. 5 shows that the spleen was essential to antibody formation in these circumstances by splenectomy 1 hour after antigenic challenge.

DISCUSSION

A cell traffic from the appendix to other lymphoid tissues was previously indicated by the work of Süssdorf (1960) and Linna, Brenning and Hemmingsson (1969). In Süssdorf's experiments the traffic of lymphoid cells from the labelled appendix was assessed on spleen imprints. By using autoradiography on tissue sections the present work has shown that this traffic is mainly directed towards follicular structures in the spleen. However, only the lymphocyte corona and the marginal zone (perifollicular envelope) are sites of migration for the appendix derived-lymphoid cells; the germinal centre compartment is excluded. Furthermore the present experiments show that the cells taking part in this appendix-to-spleen traffic are generated within germinal centres in the appendix and that this traffic does not represent a simple redistribution or recirculation of cells from the appendix to the spleen. In Linna's paper particular stress was laid on the observation that 23 per cent of his appendix-derived lymphoid cells in the spleen were detected inside germinal centres which was then interpreted as supporting the 'appendix = bursa-equivalent' hypothesis. Almost double this number (41 per cent) of labelled cells were detected in other parts of the follicular structures (lymphocyte corona + marginal zone).

The discrepancy between his finding of 23 per cent of all labelled cells localized in germinal centres and the virtual absence in the present experiments of any traffic from the appendix to germinal centres seems difficult to explain. However, in a later paper (Linna, Back and Hemmingsson, 1971) sources other than the bursa of Fabricius (or its mammalian equivalent) are suggested for the cells homing in germinal centres.

As early as 1956 the protective effect of lead shielding of the appendix as regards the antibody-forming capacity following whole body X-irradiation was demonstrated by Süssdorf and Draper (1956). They suggested that 'the protection appears to be due to the shielding of mobile cells which can repopulate the antibody-forming sites . . . .' Again, however, these data may be interpreted as merely indicating redistribution of AFCP's from the appendix to other lymphoid organs. In the appendectomy experiments described above evidence is provided for the actual generation in the appendix of AFCP's which migrate to other lymphoid organs. Comparable data were obtained by Cooper *et al.* (1966) which were then interpreted as supporting the 'appendix = bursa-equivalent' hypothesis. However, even in experiments where the whole of the gut-associated lymphoid tissue (appendix, Peyer's patches, sacculus rotundus) were removed followed by 600 rads whole body X-irradiation, they were unable to imitate a bursectomy effect as some restoration of antibody responsiveness occurred in all instances and sometimes nearly normal levels of antibody formation were observed 21 days after the irradiation. Also lymphoid tissue morphology was found to return to normal with normal numbers of plasma cells and germinal centres.

In summary, the experiments described above are believed to indicate the following: (1) a substantial cell traffic exists from the rabbit appendix to follicular structures in the spleen, i.e. to the lymphocyte corona and marginal zone, though not to germinal centres; (2) lymphoid cells taking part in this cell traffic are derived from the germinal centres in the appendix, which are essentially antigen-dependent; (3) AFCP's are generated inside the appendix. They subsequently seed out to other lymphoid tissues where upon antigenic stimulation they may transform into antibody-forming cells.

Since recovery of the capacity of the spleen to initiate an antibody response is correlated with regeneration of follicular structures in the spleen and as these latter structures, at least in part, prove to be dependent on an influx of cells derived from the germinal centre compartment of the appendix, it is suggested that the appendix as part of the gut-associated lymphoid tissue contributes to the primary antibody-forming potential of other lymphoid tissues by production of AFCP's. The latter are presumably the population of lymphoid cells derived from its germinal centres and contribute to the regeneration of follicular structures elsewhere.

Experiments described in the following paper (Nieuwenhuis and Keuning, 1974) were designed to decide whether this production of AFCP's in germinal centres of the appendix is unique to this organ or whether it is a general feature of germinal centres including those outside the gut.

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