PEYER'S PATCHES: IMMUNOLOGIC STUDIES*

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The thymus is the sole immunologic organ in the mammal which has been definitely established as being "first-order." It has been proposed that the gut-associated lympho-epithelial tissues such as the Peyer's patches are also first level lymphoid organs, performing a function analogous to that of the bursa of Fabricius in birds (1–4). This proposition is largely based on indirect evidence. Removal of the Peyer's patch from young rabbits or sublethally irradiated adult animals resulted in lowered IgM levels and an impaired ability to respond to some antigens, leaving the cell-mediated responses unaffected (1, 2). Cooper et al. suggested that the Peyer's patch represents "the site of differentiation of a population of lymphocytes with functions distinct from those of thymus derived cells." On phylogenetic grounds and from a study of the kinetics of labeling of lymphocytes within the epithelium covering the lymphoid aggregates, Fichtelius has proposed that young lymphocytes are recruited from the blood and differentiate to cells capable of immunoglobulin synthesis after interaction with the epithelial cells (3, 4). This idea would fit the general concept that the emergence of differentiated cell populations is dependent on an association between different cell types (5). In a more restricted sense the immunological maturation of a bone marrow stem cell is thought to take place under the influence of thymic epithelial cells (6–8).

First and second lymphoid organs are considered to be distinguishable by their patterns of cellular repopulation and on histological and immunological grounds. The repopulation studies of Evans et al. (9) argue against alignment of the Peyer's patch with the thymus. When they injected lethally irradiated mice with 10^5 bone marrow cells and 10^7 lymphoid cells carrying distinct marker chromosomes they observed that the thymus was almost entirely repopulated by myeloid cells, whereas the Peyer's patches, like the lymph nodes, were repopulated by lymphoid elements. In support of this conclusion is the finding that the Peyer's patches of rabbits differ from the thymus in being poorly developed at birth. The observation that gut-associated

lymphoid tissues contain a specialized epithelium and a higher percentage of dividing lymphocytes than other peripheral lymphoid organs suggests, however, that they may play a role in the genesis of immune reactivity during adult life.[1]

One expects an intrinsic difference in the ability of first and second level lymphoid organs to respond to antigenic stimuli. In vivo transfer experiments have shown that the thymus is almost completely devoid of both antibody-forming cells and the precursors of antibody-forming cells (10, 11). Such clear evidence is lacking for the Peyer's patches, and the available information is often confusing. Stoner and Hale (12) demonstrated that Peyer's patch tissue from hyperimmunized mice could mount a secondary response when implanted intraocularly in irradiated animals. Under their experimental conditions, however, thymic tissue was also competent. No specific antibody-containing cells of any immunoglobulin class could be detected by immuno-fluorescence in the Peyer's patches of rabbits which had been hyperimmunized with ovalbumin by a variety of parenteral routes. Recently a study on the immunological capabilities of rat gut lymphoid tissue after local immunization has pointed up the danger of relying on humoral assays and the difficulties in interpreting results obtained with in vivo stimulation because of the migration of both antigen and cells (13, 14).

In our studies of the immunological capabilities of the Peyer's patch of the rabbit, we have done some in vivo experiments to ascertain the presence of specific antibody-forming cells following stimulation with sheep red cells, since the hemolytic plaque technique enabled us to rapidly test the whole organ for such cells. However, to avoid both the uncertainties associated with stimulation studies in vivo and the possible contribution of the irradiated host, always an unknown in transfer studies, we have also directly tested the capabilities in the in vitro system devised by Mishell and Dutton (15) for studying the response of mouse spleen suspensions to heterologous red cells. We have looked at the response to sheep red cells of dissociated cell suspensions from the Peyer's patches, the spleen, and the thymus of both normal and stimulated rabbits. The cells derived from the thymus and the spleen were considered to be prototypes of first and second level lymphoid organs.

Materials and Methods

Animals.—Mature male New Zealand white rabbits were used.

Immunization.—For intravenous and intraperitoneal injections we used 1.0 ml containing 5.10^9 washed sheep red cells (SRC),[2] a dose which gives a maximal plaque-forming cell (PFC) response in the rabbit spleen 4 days after a single injection. We used 4 groups of stimulated rabbits: groups 1 and 2 were killed 4 and 8 days respectively after a single injection of 5.10^9 SRC, groups 3 and 4 were killed 4 and 7 days respectively after 2 injections of 5.10^9 SRC, spaced a week apart.

Direct injection of the Peyer's patch was done with a 27 gauge needle as described previ-

[1] Faulk, W. P., J. M. McCormick, J. Goodman, J. M. Yoffey, and H. H. Fudenberg, 1969. Peyer's patches: morphologic studies. Unpublished results.

[2] *Abbreviations used in this paper:* DPFC, direct plaque-forming cells; IDPFC, indirect plaque-forming cells; PFC, plaque-forming cells; SRC, sheep red cells.

ously.[1] Although 80 µl, containing 8.10^8 SRC, was delivered, we observed considerable back flow so that only a small fraction of that amount arrived in the patch. Successful injections were indicated by an immediate reddening of the patch.

Enumeration of Antibody-Forming Cells.—The hemolytic plaque technique which was used to score cells synthesizing antibodies to SRC (PFC) has previously been described in detail (16, 17). Unless otherwise stated, we scored only for direct PFC (DPFC), or for those releasing IgM antibodies. To develop indirect PFC (IDPFC), or those due to IgG, we used goat anti rabbit γ-globulin[3] at a dilution of 1:800. At this dilution the antiserum was not inhibitory for DPFC so that the number of IDPFC was determined by subtracting the number of plaques obtained in the absence of antiserum. Responses are expressed as PFC per 10^6 lymphoid cells.

In Vitro Stimulation.—Each rabbit was killed with a minimal amount of Euthanol-6[4] and cultures were prepared from the spleen, the thymus, and 4–6 pooled Peyer's patches. Care was taken to avoid excessive contamination with the blood, and in the case of the thymus, with neighboring lymph nodes. Both the method for preparation of the cell suspensions and the culture conditions were those described by Mishell and Dutton (15) for mouse spleen cells, with two minor additions—the culture medium contained 100 units of penicillin and 100 µg of streptomycin per ml, and the nutritional mixture for daily feeding also included 2.5 ml of 100 × MEM vitamins (Microbiological Associates, Inc., Bethesda, Md. 13–607) per 56 ml. Control cultures (C) contained 1.5 to 2.10^7 lymphoid cells. Test cultures (T) contained in addition 8.10^6 SRC. Two Petri dishes from each C and T series were pooled daily from the 2nd to the 6th day of culture and assayed for PFC and the number of surviving cells Responses are generally expressed as PFC per 10^6 recovered cells.

RESULTS

In Vivo Stimulation

It has been repeatedly shown that most tissues from animals which have never been exposed to SRC nevertheless contain background PFC. It was therefore necessary to establish this level for the Peyer's patch so that we could assess the significance of levels found in stimulated rabbits. The variation between individual animals is characteristically large (the distribution in mouse spleens fits a negative binomial distribution, or approximates a log normal.[5]), but it is apparent from Table I that the levels in the Peyer's patch are significantly below those of the spleen or other gut-associated lymphoid organs. It has been suggested that background PFC may arise spontaneously since they are found in mice and chicks within a week of birth,[6] and in germ-free mice maintained on an antigen-free diet (18). More commonly they are presumed to be the result of exposure to cross-reacting enteric bacteria (19, 20) an idea consistent with the presence of high PFC levels in the appendix. The low levels in the Peyer's patch may be due to a variety of factors, e.g., that its lymphocytes are less capable of responding, are uniquely protected from gut antigen, or migrate rapidly to other tissues.

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[3] Kindly provided by Dr. H. O. McDevitt, Department of Medicine, Stanford University Stanford, Calif.

[4] Trico Pharmaceutical Co., Oregon City, Oreg.

[5] D. Kodlin, 1965. Unpublished observation.

[6] H. Fuji, 1965. Unpublished results.

In the Peyer's patches of animals which had received one or two injections of SRC, we detected an increase in cells forming specific antibody of both IgM and IgG classes (Table II). A number of observations have led us to conclude that these new PFC have not arisen in situ but are immigrants from the spleen. (a) Only one animal in group 1, where the spleen shows a peak IgM response, has a Peyer's patch response outside the normal range. At later times when PFC appear in large numbers in the blood, all animals in groups 2–4 show an increased level of PFC in the Peyer's patches. (b) There is a reasonably good correlation between the magnitude of both the spleen DPFC and IDPFC re-

TABLE I

Background PFC Level per 10^6 Lymphoid Cells in Unstimulated Rabbits

Organ	Arithmetic mean	Range	No. of animals
Peyer's patch	0.2	0.04–0.36	15
Appendix	2.3	0.26–5.5	10
Sacculus rotundus	1.2	0.2–1.29	7
Spleen	1.2	0.1–3.1	15
Thymus	0.02	<0.002–0.092	12

TABLE II

In Vivo Stimulation: The PFC Response* per 10^6 Lymphoid Cells of Stimulated Rabbits

Organ	Group 1	Group 2	Group 3	Group 4
Spleen	663	240 (45)	550 (103)	328 (356)
Peyer's patch	1.6	4 (0.9)	14.3 (5.1)	5.2 (15.2)
Thymus	0.01	0.15 (04)	0.58 (—)	1.1 (—)

Groups 1 and 2 were killed 4 and 8 days respectively after a single injection of 5.10^9 SRC. Groups 3 and 4 were killed 4 and 7 days respectively after 2 injections of 5.10^9 SRC, spaced a week apart. Each group contains 4 to 6 rabbits. The values are the arithmetic means.

* Unenclosed figures represent DPFC; those in parentheses represent IDPFC.

sponse of individual animals and those of the corresponding Peyer's patches. Since the $t_{1/2}$ of peripheral PFC in the rabbit must be greater than the 14 hr found for mouse peripheral PFC,[7] the correlation could not be expected to be perfect.

Since the seeding of the Peyer's patches with antibody-forming cells aligns them with secondary lymphoid organs, the most obvious explanation for the failure of the Peyer's patches to respond to SRC is that the intravenous injection does not expose the antigen-sensitive cells to antigen. Neither intraperitoneal injections of SRC nor feeding lysed red cells for 4 days improved the response. Injection into the Peyer's patch vein did not affect the levels of PFC in

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[7] Henry, C., and A. A. Nordin, 1965. Unpublished results.

the gut-associated lymphoid tissues though the spleen PFC response increased to 400 PFC per 10^6 cells. This finding is perhaps not unexpected; the spleen has in its pulp a trapping device for holding up red cells, but there is not evidence of anything like this in the Peyer's patch. Although the SRC redden the patch at the time of injection, they are probably washed out almost immediately by the blood flow and end up in the spleen.

In Vitro Stimulation

The Response of Cells from Unprimed Rabbits.—The Peyer's patch cells of the rabbit are not intrinsically incapable of responding to SRC as is shown by their behavior following in vitro challenge (Table III). In 12 attempts we always obtained a response from Peyer's patch cultures; with spleen cells we were successful on 9 occasions, while thymus cells never responded. Since this is the first reporting of in vitro stimulation of cells from a source other than mouse spleen,

TABLE III

In Vitro Stimulation: Responses* of Cultures Prepared from Unstimulated Rabbits

Source of cells	Control cultures	Test cultures
Peyer's patch	3.7 (1–17)	62 (22–134)
Spleen	18 (4–58)	140 (28–475)
Thymus	<0.1	<0.1

* Responses are given as PFC per 10^6 recovered cells on the 5th day of culture. They represent the mean of 12 experiments. The range of responses obtained is given in parentheses.

a number of points merit comment. (*a*) The kinetics of the response of both the Peyer's patch and spleen cultures were similar to those described by Mishell and Dutton (15). There was a lag period of 1–2 days, and the peak response was always observed on the 5th day of culture. (*b*) The peak response, which was about one-third of that obtained with mouse spleen cells, was not improved by changes in the culture conditions. (*c*) High responses were often noted in the absence of antigen, particularly in the rabbit spleen C cultures, and the C response was not correlated with the height of the corresponding T response. Since addition of 0.05 μg of the antimitotic agent, vinblastine, to each culture inhibited the C responses, they are not due to a population of cells similar to that found in mouse peritoneal exudates (21, 22). Apparently rabbit spleens contain a large number of cells which are sensitive to the antigen in fetal calf serum which cross-reacts with SRC antigens (15). (*d*) The cell survival in the Peyer's patch and thymus cultures was superior to that of the spleen cultures— 80–90% of the initial cells were recovered after 5–6 days of culture, compared with 30–40% from the spleen cultures. In Table III, where the results are expressed as PFC per 10^6 recovered cells, the PFC responses of Peyer's patches

appear slightly inferior to those of spleens. This difference is perhaps artificial since it disappears when the results are expressed as PFC per 10^6 cells initially seeded.

In view of the findings that the kinetics of the response of spleen and Peyer's patch cultures are identical, and that the proportion of cells which can respond to SRC in the two types of culture is similar, it is difficult to imagine that the competence of the Peyer's patch is limited to the lymphocytes which are in association with the epithelium lining the lumen. However, as a direct test of Fichtelius' proposition (3, 4), we carefully stripped off most of the epithelia from half of the patches of one rabbit, using a dissecting microscope and keeping the tissues at 4°C. Cultures prepared from three stripped and three unstripped patches were then compared for their ability to mount a primary in vitro response. Table IV, which gives the results of two such experiments, strongly suggests that epithelial cells are not required for this response.

TABLE IV

Removal of the Peyer's Patch Epithelium. The In Vitro PFC Response of Stripped and Unstripped Cultures

Animal number	Treatment	Maximal response* per Petri dish
1	Unstripped	532 (10)
	Stripped	566 (6)
2	Unstripped	439 (26)
	Stripped	323 (9)

* Both stripped and unstripped cultures contained 1.8×10^7 cells. The control values are given in parentheses.

The Response of Cells from Primed Animals.—Cultures prepared from rabbits which have received one or two injections of SRC show a number of characteristics which distinguish them from cultures prepared from normal animals. The response to SRC is several hundredfold higher, and the kinetics of the response are different. Cultures of primed animals achieve their peak response at 3 or 4 days, compared with 5 days for unprimed cultures. In addition, the response can be evoked under culture conditions adverse to the development of a primary in vitro response. Brief incubation at an alkaline pH during the first 2 days of culture generate conditions which permit a secondary in vitro response but do not support a primary response. We could thus differentiate a response due to memory cells from one due to "virgin" antigen-sensitive cells. Using such an assay system, we have demonstrated the presence of IgM memory cells in the spleen of rabbits which had received an intravenous injection of 5.10^9 SRC 3 days previously, the earliest time at which we have tested a primed rabbit. The Peyer's patches of these animals showed no evidence of memory cells. IgM memory in these tissues was acquired at later times, coincident with the acquisi-

tion of PFC. In Fig. 1 we have plotted the 3 or 4 day response of spleen and Peyer's patch cultures from the primed rabbits in groups 2, 3, and 4 against the PFC level present before restimulation in vitro. Some thymic cultures showed slight responses (0.6–9.6 per 10^6 recovered cells), but the level of the thymic T series rarely exceeded those of the corresponding C series, and they are too low to be included in Fig. 1. Both lines in the figure have slopes of 1. If one considers only the responses of the spleen cultures (the filled symbols) one notes that a reasonably good correlation occurs between the height of the response and the
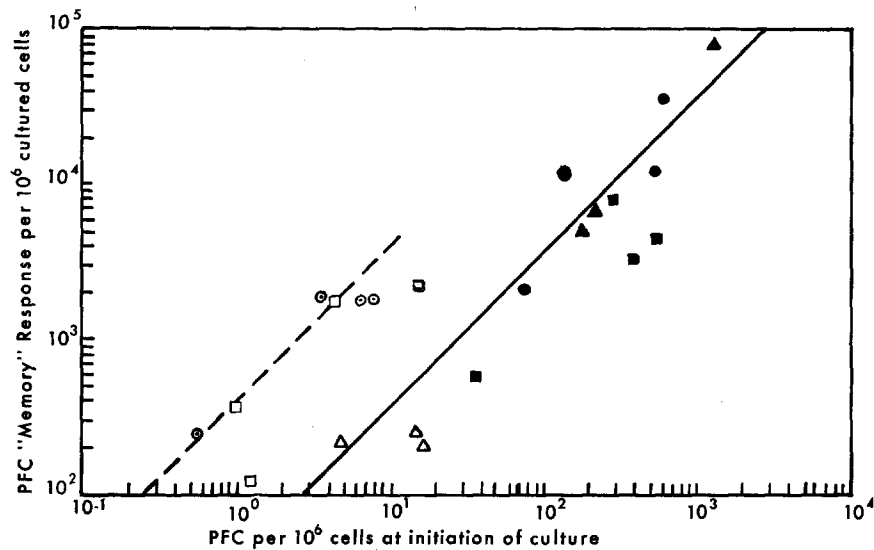

FIG. 1. Maximal PFC responses of cultures of spleen (filled symbols) and Peyer's patch (open symbols) from primed rabbits. The squares represent rabbits killed 8 days after a single injection of 5.10^9 SRC. The triangles and circles represent animals killed 4 and 7 days respectively after two injections of 5.10^9 SRC, spaced a week apart.

initial level of PFC, and that the splenic cultures from rabbits of groups 3 and 4 respond to new antigen in a very similar manner. Since the spleen is the main site of stimulation following intravenous injection (23), it appears that memory cells are generated in the same process that generates PFC. The property of IgM memory is not carried by the PFC themselves, however, as seen from a consideration of the responses of the Peyer's patch cultures. Although those prepared from animals which had received the second of two injections 4 days previously behave like spleen cultures, the responses of cultures prepared 3 days later are seen displaced tenfold to the left, showing increased responsiveness per preexisting PFC. During the 3 days, the Peyer's patches apparently acquired new memory cells but no new PFC.

In some spleen and Peyer's patch cultures of primed rabbits we have found an increase in IDPFC, but this increase is neither as consistent nor as spectacular as that of the DPFC. At the peak of the in vitro response of primed spleen cultures we have found that up to 10% of the recovered cells can score as DPFC, while we have never found an IDPFC response which exceeded 6000 IDPFC per 10^6 cells. This aspect, which is probably due either to our culture conditions or our antiglobulin serum, is being further investigated.

DISCUSSION

Recent observations suggest that the primary immune response to SRC requires the cooperation of two or three cell types (10, 11, 24, 25), thymus and marrow-derived lymphocytes and possibly also a macrophage population. All the requisite types appear to be present in the spleen, the lymph nodes, and the thoracic duct lymph. In contrast, the thymus and marrow each lack at least one type. Our finding, that cultures of Peyer's patches from nonprimed rabbits can respond in vitro to SRC, shows that these tissues also contain the necessary cell populations. The response was shown to be independent of the presence of epithelial cells which cover the lymphoid aggregates, and moreover, is very similar to that of spleen cultures from normal rabbits. In contrast, thymic cultures cannot mount a primary in vitro response. We feel that these findings place the Peyer's patch in the category of secondary lymphoid organs as these are currently defined. Recently, Armstrong et al. (26), using a transfer system to score for cells responsive to polymerized flagellin, an antigen with different cellular requirements for "recognition," arrived at the same conclusion.

Pierce (27) has shown that lymphoid cells from primed mice can respond to SRC in vitro in macrophage-poor cultures while similar cultures from nonprimed mice cannot. It may be that our modified culture conditions, which will support a secondary but not a primary in vitro response, adversely affect the macrophages.

The following description most simply explains our results with primed tissues. After stimulation in the spleen, IgM memory cells appear rapidly in the same process that generates PFC. Both PFC and memory cells are then seeded to other lymphoid organs, including the Peyer's patches. Since the memory response of the spleen cultures is always several hundredfold higher than that of the Peyer's patches, the majority of memory cells evidently remain at the site of the initial stimulus, a situation proposed for long-lived memory to protein antigens (28). The observation that the ratio of memory response to PFC of the Peyer's patch cultures increases with the interval after the last injection indicates that the properties of IgM memory and plaque formation do not necessarily coexist, but does not exclude the possibility that PFC may also be memory cells. The acquisition of memory cells without a concomitant increase in PFC suggests that memory cells may either divide in the patches, be

selectively and continuously seeded, or arise by differentiation of the PFC. In the thymuses of primed rabbits we have found a small number of both PFC and cells which can respond to SRC in vitro. We cannot exclude the possibility that the low activities are due to blood-borne cells.

The gut-associated lymphoid organs represent large aggregates of lymphocytes with high mitotic activity ([29] and footnote[1]). Our experiments do not clarify the role these lymphocytes play in the response of animals to foreign antigens. We were not successful in stimulating these cells in vivo by any course of injections of SRC. Cooper et al. (13) have reported that placement of bacterial antigen in the lumen of an isolated intestinal loop containing a Peyer's patch caused generalized reticuloepithelial stimulation, but no antibody production, by the intestinal lymphoid cells. A local response was only observed when antigen was directly inoculated into Peyer's patches, but even then the response was tenfold inferior to that of the draining mesenteric node (14). Considering this success with an injection procedure which cannot be duplicated in nature, and our in vitro experience, it seems most likely that the antigen-recognizing cells in these organs cannot make contact with antigen which is given either parenterally or placed in the gut lumen. The possibility that large numbers of cells synthesize an antibody of an immunoglobulin class which is inefficient in conventional serological tests seems excluded by the immunofluorescent results.[1] The most likely candidate of this sort would seem to be IgA. We have also tested for this possibility in mice injected with SRC, using two samples of anti-mouse IgA,[8] but have been unable to detect any PFC due to IgA anti-SRC antibodies. Our in vitro experiments with primed animals show that the Peyer's patches receive memory cells generated by a splenic stimulus. The gut-associated lymphoid tissues thus appear to be a fruitful source of antigen-reactive cells which may migrate to other sites to be stimulated. One could imagine that this role is fulfilled by the circulating small lymphocytes, and that the mitotically active large lymphocytes, which migrate through the lymphoid follicles towards the epithelium,[1] endow the Peyer's patches with another function. If lymphopoiesis in the gut tissues is as wasteful as these results suggest, they may well be sites which function in adult life to eliminate "forbidden" clones and to generate antibody diversity (30).

SUMMARY

The immune capabilities of the Peyer's patches have been investigated by the use of an in vitro system.

Despite our failure to stimulate Peyer's patch lymphocytes in vivo it appears that Peyer's patches behave immunologically as peripheral lymphoid tissues.

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[8] Kindly provided by Dr. A. A. Nordin, Lobund Laboratories, Notre Dame, Ind., and Dr. H. M. Grey, Scripps Clinic and Research Foundation, La Jolla, Calif.

Cultures prepared from the dissociated Peyer's patches of normal rabbits respond to sheep erythrocytes. The response is comparable to that obtained with spleen cultures from the same animals and is not dependent on the presence of the epithelial cells which line the lumen. Similar thymic cultures do not respond.

Our experiments with cultures prepared from rabbits which have received one or two injections of SRC show that the Peyer's patches contain both IgM and IgG "memory" cells which have migrated from the spleen. The concentration of these cells in the spleen remains several hundredfold higher.

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