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Non-random distribution of intraepithelial lymphoid cells in follicle-associated epithelium of Peyer's patches in mice

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INTRODUCTION

Peyer's patches are aggregations of lymphatic nodules in the intestinal mucosa and submucosa. Each lymphatic nodule is associated with a dome, covered from the luminal side by a 'dome epithelium' or 'follicle-associated epithelium', the structure specific for this anatomical site. Intestinal villi and crypts surround the domes of Peyer's patch.

The intraepithelial lymphoid cells occur both in the villus and dome epithelium (Ferguson, 1977), and are especially numerous in the latter (Faulk *et al.* 1970; Ferguson & Parrott, 1972; Owen & Jones, 1974; Abe & Ito, 1977, 1978; Chu, Glock & Ross, 1979; Smith, Jarvis & King, 1980).

The epithelium of the domes of Peyer's patches may serve special immunological functions, since it contains both lymphoid and characteristic M (microfold or membranous) cells (Owen & Jones, 1974; Owen, 1977; Chu *et al.* 1979; Smith *et al.* 1980), which are capable of transporting antigens from the intestinal lumen into the subepithelial lymphoid tissue (Bockman & Cooper, 1973; Owen, 1977; Wolf *et al.* 1981). The M cells, therefore, may facilitate contact between the lymphoid cells and the antigens present in the intestinal lumen, while preserving the integrity of the epithelial lining.

On the other hand it has been proposed that lymphocytes migrating into the dome epithelium of Peyer's patches may induce differentiation of the absorbing epithelial cells into M cells (Smith & Peacock, 1980).

In the light of recent interest in the follicle-associated epithelium of Peyer's patches and the intraepithelial lymphoid cells the present quantitative study has been undertaken of the lymphoid cells within the epithelium, with particular attention to their random versus non-random spatial distribution. No such study has been published hitherto, although a grouping of intraepithelial lymphocytes has been suggested on the basis of visual assessment of histological specimens (Faulk *et al.* 1970; Chu *et al.* 1979). For comparison, lymphoid cells within the epithelium of villi adjacent to Peyer's patches have also been studied quantitatively.

In order to determine the pattern of distribution of lymphoid cells within the epithelium, two statistical procedures have been applied: fitting the Poisson distribution to the observed distribution of lymphoid cells per unit segment of epithelium and the test of randomness for a series (runs) of lymphocyte-rich segments of epithelium.

MATERIALS AND METHODS

Animals

Adult Balb/C and CFW/L1 mice, both sexes, kept on a standard laboratory diet, were used throughout the study. Seven Balb/C and five CFW/L1 mice were used. Animals were anaesthetised with ether and killed by cervical dislocation.

Histological procedures

The small intestine was exposed, and one to three jejunal Peyer's patches from each mouse were excised and placed immediately in fixative consisting of 2% glutaraldehyde and 2% formaldehyde in 0·1 M phosphate buffer, pH 7·4. After fixation for three hours at room temperature, the tissues were washed in the same buffer and post-fixed in 1% OsO_4 in 0·1 M veronal-acetate buffer, pH 7·4, for one hour at room temperature. After rinsing the samples, they were stained *en bloc* with 0·5% uranyl acetate in veronal-acetate buffer, pH 5·6, dehydrated in ethanols and propylene oxide, and embedded in Epon 812.

Sections 1·5 μm thick were cut with a Tesla BS-490A ultramicrotome and stained with 1% toluidine blue.

Tissue sampling

Histological sections through the centre of each Peyer's patch were used for quantitative analysis. Profiles of individual follicles with their associated intestinal epithelium were selected from these sections. Only those profiles in which the intestinal epithelium covering the follicle was cut perpendicularly to the surface of the epithelium were included for analysis. The criterion for perpendicularity was the occurrence of a single layer of epithelial cell nuclei. Fragments of dome epithelium cut obliquely, or containing artifacts, were not included.

Similar criteria applied to the epithelium covering intestinal villi. The villi included in the quantitative analysis were those situated within the region of Peyer's patches and adjacent to the lymphatic follicles.

Selected regions of histological sections of Peyer's patches were photographed, printed at a final magnification of $\times 680$, and montages constructed of the full individual profile of the dome (Fig. 1).

Seven mice of the Balb/C strain were used. Ten patches (one or two from each mouse) were included in the analysis, yielding a total of 12 profiles of dome epithelium suitable for analysis by the criteria given above. The length of individual profiles varied from 198 μm to 792 μm. The data for 12 profiles were pooled and resulted in a sample length of 5907 μm of dome epithelium. A total of 612 lymphoid cells was counted in this sample.

Ten Peyer's patches from five CFW/L1 mice were included in the quantitative analysis (one or three Peyer's patches from each mouse). In these patches, a total of 14 profiles of follicles with a dome epithelium were suitable for analysis. The pooled length of follicle-associated epithelium was 6281 μm, the individual profile length varying from 132 μm to 715 μm, and 599 lymphoid cells were counted.

Similarly, quantitative data acquired from individual profiles of villi adjacent to Peyer's patch domes were combined to form a pooled sample for the respective strains of mice. For Balb/C mice the pooled sample was 5709 μm of villus epithelium

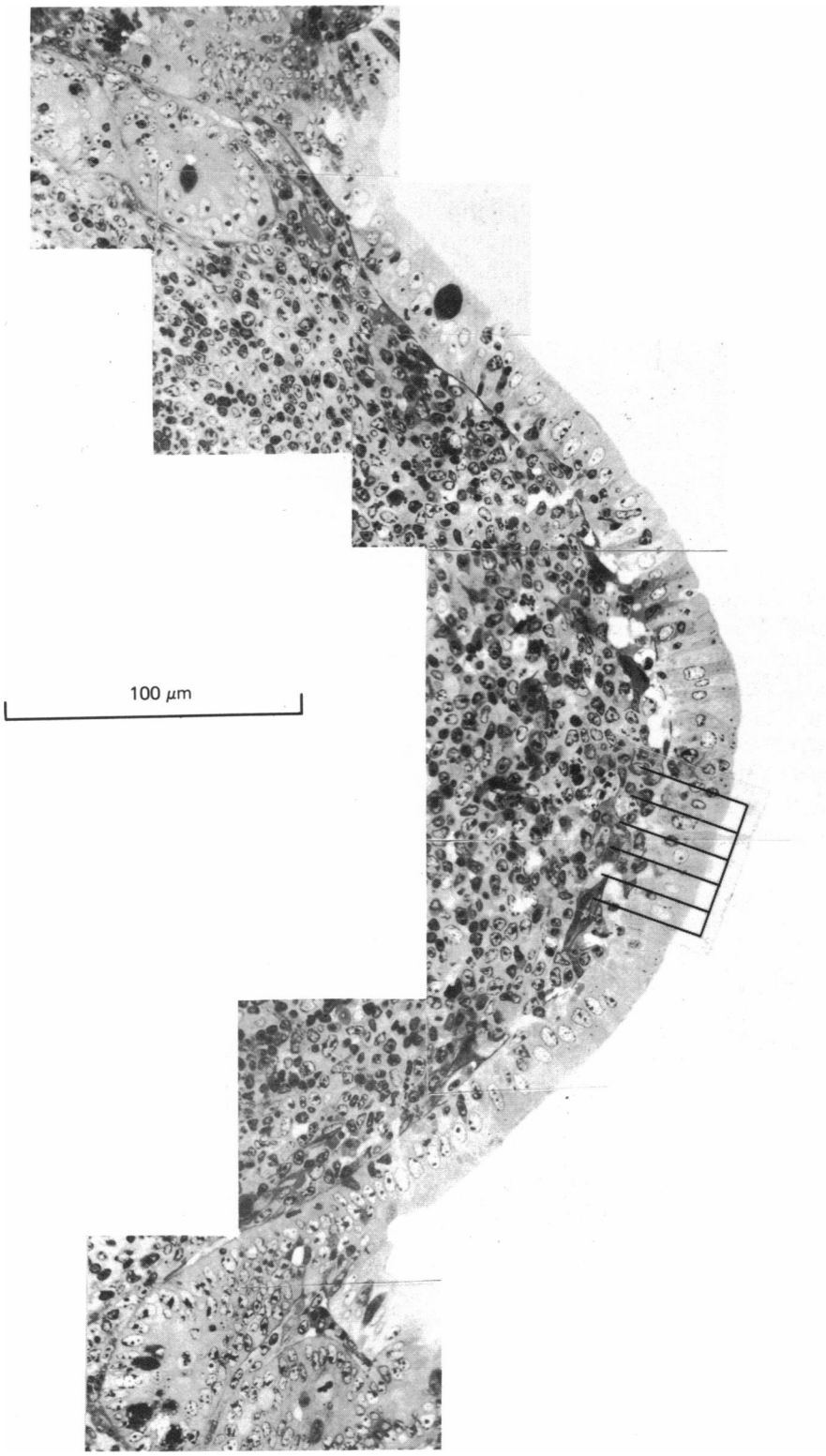


Fig. 1. A montage of the dome of a Peyer's patch in a CFW/L1 mouse. Grid of unit segments superimposed over a fragment of the follicle-associated epithelium. Follicle-associated crypts with Paneth cells are seen at both ends of the dome epithelium. ×400.

containing 179 lymphoid cells. For CFW/L1 mice the sample size was 6314 μm of the epithelium and 146 lymphoid cells.

The sampling described above yielded a roughly equal length (about 6000 μm) of follicle-associated epithelium and villus epithelium for each of the two strains of mice.

Histometric analysis

Histometric analysis of the distribution of lymphoid cells within the intestinal epithelium was performed by counting the number of lymphoid cell nuclei within the segments of a special grid superimposed on the prints (Fig. 1). Lymphoid cells situated on a grid bar were assigned to the segment containing the centre of the cell nucleus. The grid consisted of oblong segments, each 11 μm wide. The width of a segment was equal to the average distance between two neighbouring epithelial cell nuclei in a follicle-associated epithelium, as determined by preliminary measurements. The long axis of the grid was superimposed parallel to the apical border of the epithelium (Fig. 1), its orientation changing according to the curvature of the epithelium. An identical procedure was used for analysis of villus epithelium.

For the purposes of this study, lymphoid cells comprised both typical small lymphocytes with round, dense nuclei and scanty cytoplasm, as well as cells with large nuclei and moderately abundant, lightly stained cytoplasm. Plasma cells were also included in this category. Epithelial cell nuclei were distinguished from the above by their oval shape, large size and pale nuclear chromatin with few chromocentres (Fig. 2). Small, unclassifiable nuclear fragments were ignored.

Statistical procedures

The numerical data were presented in the form of frequency distributions (histograms) of the number of lymphoid cell nuclei per grid segment (Figs. 3, 5).

Theoretical Poisson distributions were generated by a computer programme with the parameter equal to the mean number of lymphoid cells per grid segment as determined by histometric analysis, for dome and villus epithelium respectively.

The goodness of fit between the theoretical Poisson distribution and the observed distribution was tested by the chi-square test.

The 'test of randomness for runs' (Freund, 1967) was used to test whether segments of epithelium containing lymphoid cells occurred in clusters or were distributed at random. For this purpose, the segments of dome epithelium were classified into three categories: (*a*) containing more than one lymphocyte per segment (i.e. above the median), labelled as positive; (*b*) containing no lymphocytes (i.e. less than the median), labelled as negative; (*c*) containing one lymphocyte per segment (i.e. equal to the median value). The segments containing the median number (one) of lymphocytes per segment were neglected, as appropriate for the test of randomness for runs above and below the median (Freund, 1967). A run was defined as a group of neighbouring segments labelled with the same sign (either positive or negative; see Figure 4) and the number of positive and negative runs was determined. The observed number of runs was compared to the number predicted on the assumption of a random distribution of positive and negative segments (Freund, 1967). The test of significance was based on the standardised normal distribution (Freund, 1967).

A similar test was applied to the distribution of lymphocyte-containing segments in villus epithelium. In this case only two categories of segments were distinguished, namely, negative segments with no lymphoid cells, and positive segments with one

or more lymphoid cells. The rest of the procedure was the same as that for the dome epithelium.

In this statistical analysis, a significant deviation of the observed number of runs from the theoretical value calculated from the data for a given sample indicated non-randomness. A positive deviation (number of observed runs greater than number of predicted runs) indicated an even or alternating distribution, while a negative deviation was interpreted as produced by clustering. There is no significant difference between the observed and theoretical number of runs when the sequence of positive and negative elements in a sample is random (Freund, 1967).

The significance of the differences between the mean numbers of lymphoid cells per segment (in Balb/C versus CFW/L1 mice and in follicle-associated epithelium versus villus epithelium) was tested by the *t*-test. Since the frequency distribution of the variable was not normal but Poisson in type, the original data were transformed into the $\sqrt{(x+1)}$ scale in order to stabilise the variances, as suggested by Snedecor & Cochran (1974). The *t*-test was performed on this transformed data.

All computations were performed on a Hewlett Packard HP-9821-A desk computer, using programmes designed by the first author.

RESULTS

Qualitative observations

Inspection of histological sections through Peyer's patches revealed that intraepithelial lymphoid cells were apparently more numerous in follicle-associated than in villus epithelium (Fig. 2). The intraepithelial lymphoid cells of the villi were scattered along the epithelium as single elements or occasionally in pairs; most of them were situated close to the basement membrane. By contrast, the lymphoid cells within follicle-associated epithelium often occurred in groups containing several cells (Fig. 2); the lymphoid cells were situated at all levels of the epithelium, some of them close to its luminal border.

No preferential location of accumulations of intraepithelial lymphoid cells was noted with respect to the apex of the dome. The accumulations were scattered throughout the apices, slopes and bases of the domes adjacent to the follicle-associated crypts.

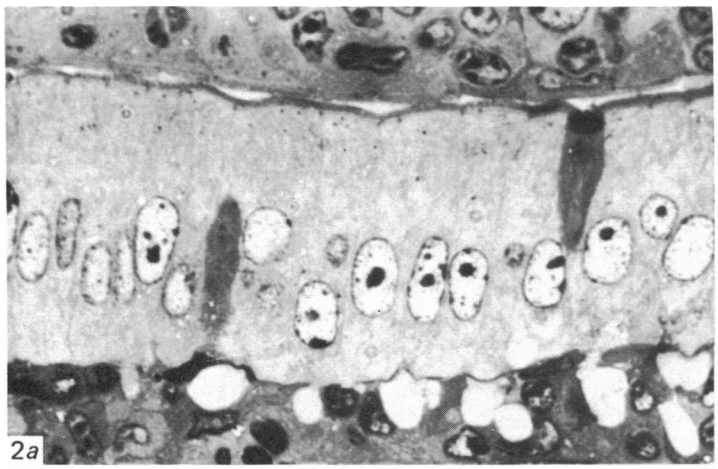
Grouping of the lymphoid cells, although suggestive, could not be judged as non-random by observation alone.

Size and morphological appearance of the intraepithelial lymphoid cells varied but they were distinguished from epithelial cells mainly by the smaller size of their nuclei and by a more dense and more granular chromatin (Fig. 2*b*). Within the class of lymphoid cells, plasma cells were recognised by a strongly basophilic cytoplasm and a round, dense nucleus.

In areas of follicle-associated epithelium, where many intraepithelial lymphocytes were present, an irregular arrangement of the epithelial cell nuclei was observed. Conversely, areas containing few lymphocytes (Fig. 2*c*) showed a regular pattern of epithelial cell nuclei, located in the middle of the epithelium, similarly to the villus epithelium (Fig. 2*a*).

The M cells were not identified because the criteria for identification depended on ultrastructural features requiring electron microscopy.

In several instances, the interface between the follicle-associated epithelium and the underlying connective tissue was difficult to trace, particularly in regions of accumulations of intraepithelial lymphoid cells.



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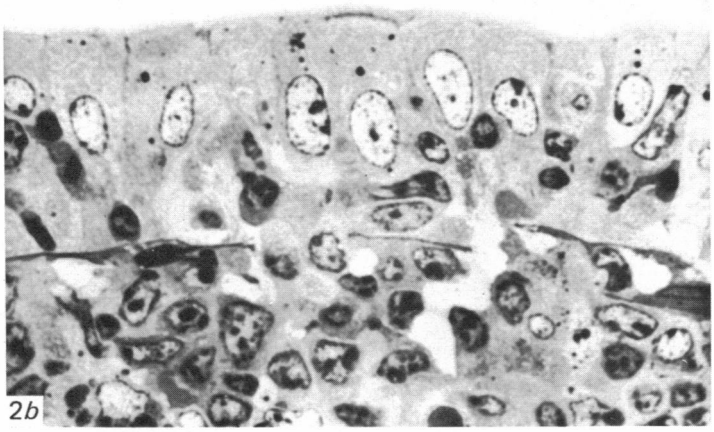

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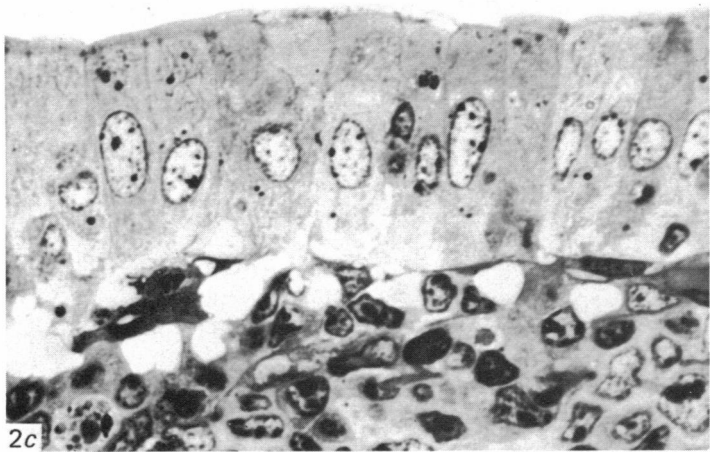

2c

No difference in morphology, number and distribution of the intraepithelial lymphoid cells was noted between Balb/C and CFW/L1 mice.

Number and distribution of intraepithelial lymphoid cells in the dome epithelium

The mean number of lymphoid cells per segment of the dome epithelium was 1·14 ± 1·19 (S.E.M.) in Balb/C mice and 1·05 ± 1·11 in CFW/L1 mice. The difference was not significant using the *t*-test ($P > 0{\cdot}05$).

The frequency distribution of the intraepithelial lymphoid cells per segment of dome epithelium was skewed to the right (Fig. 3) with the maximum at zero both in Balb/C and CFW/L1 mice. The largest number of intraepithelial lymphoid cells per segment was seven: one such segment was found in a sample from Balb/C mice. The median value both in Balb/C and CFW/L1 mice was one lymphocyte per segment.

The theoretical Poisson distributions generated for the observed means ($m = 1{\cdot}14$ and $m = 1{\cdot}05$, for Balb/C and CFW/L1 mice respectively) differed from the observed distributions. In both strains of mice, the numbers of segments containing 0, 3, 4 and 5 lymphoid cells were larger in the observed than in the predicted Poisson distributions, while the numbers of segments with one or two lymphoid cells were smaller in the observed than in the theoretical distributions (Fig. 3). Using the chi-square test, the observed were significantly different from the theoretical Poisson distributions ($P < 0{\cdot}001$, degrees of freedom = 4 for Balb/C mice; $0{\cdot}01 > P > 0{\cdot}001$, D.F. = 4 for CFW/L1 mice). The frequency distribution in Balb/C mice was not significantly different from that in CFW/L1 mice ($0{\cdot}25 > P > 0{\cdot}1$, D.F. = 4).

Grouping of segments rich in lymphoid cells, suggested by histological observations of dome epithelium, was tested objectively by the test of randomness for runs above and below the median (Fig. 4).

The number of runs (total of positive and negative runs) in the sample of 537 segments in Balb/C mice was 105 which was significantly smaller than the expected random value of 142 runs ($P < 0{\cdot}001$). In a sample of 571 segments in CFW/L1 mice, there were 140 runs while the expected random value was 191; the two values were significantly different ($P < 0{\cdot}002$). Therefore, in dome epithelium, grouping of segments rich in lymphoid cells and of segments depleted of lymphoid cells is a real phenomenon in both strains of mice.

Number and distribution of intraepithelial lymphoid cells in the villus epithelium

The mean number of intraepithelial lymphoid cells per segment of villus epithelium was 0·34 ± 0·29 (S.E.M.) in Balb/C mice and 0·25 ± 0·48 (S.E.M.) in CFW/L1 mice. The difference between the two strains was not significantly different ($P > 0{\cdot}8$).

The frequency distribution of the intraepithelial lymphoid cells per segment of villus epithelium was again skewed to the right (Fig. 5), with the maximum at zero, in both Balb/C and CFW/L1 mice. No segments with more than three lymphocytes were found in either strain. The observed distributions were not significantly different from the theoretical Poisson distributions predicted for the observed means ($m = 0{\cdot}34$ and $m = 0{\cdot}25$ respectively) as tested by the chi-square test: $0{\cdot}25 > P > 0{\cdot}1$,

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Fig. 2(*a–c*). The intestinal epithelium in the region of an ileal Peyer's patch in a CFW/L1 mouse; (*a*) the villus epithelium; (*b*) the follicle-associated epithelium with accumulation of lymphoid cells; (*c*) the follicle-associated epithelium depleted of lymphoid cells. × 1500.

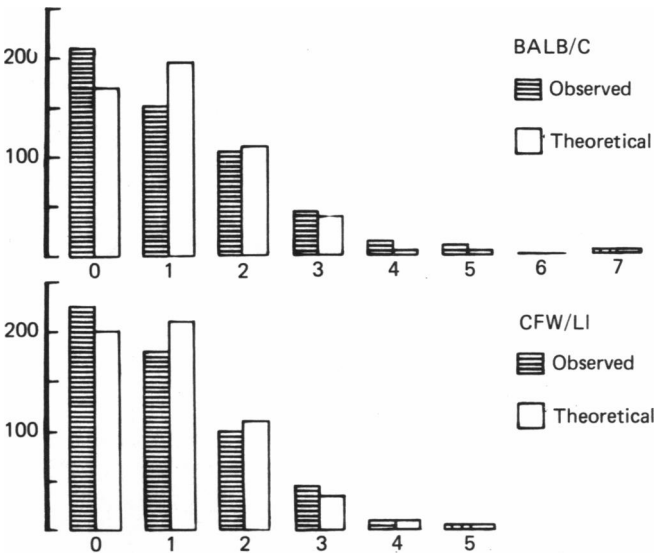

Fig. 3. The observed frequency distribution of the numbers of lymphoid cells per segment of follicle-associated epithelium (dome epithelium) of Peyer's patches in two strains of mice (hatched histograms). The theoretical Poisson distributions, fitted to the observed means, are presented as white bars on the histograms. The observed and theoretical distributions are significantly different for both strains. Vertical scale: frequency (number of segments); horizontal scale: number of lymphoid cells per segment.

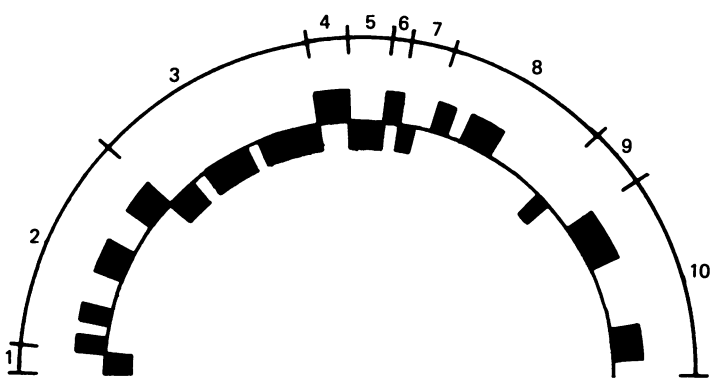

Fig. 4. The diagram illustrates the distribution of lymphocyte-rich and lymphocyte-depleted runs of segments in the dome epithelium of Peyer's patch. The bars above and below the inner semicircular line denote segments of epithelium with more than one lymphoid cell, and with no lymphoid cells, respectively. The segments containing one lymphoid cell (a median value) are indicated by the absence of a bar. The division of epithelium into runs is shown on the outer curvature, the runs (series) being consecutively numbered from 1 to 10. The number of runs for this small sample, expected on the assumption of a random distribution of lymphocyte-rich and lymphocyte-depleted segments, is about $15 \pm 2{\cdot}7$. The data for this diagram are taken from one section of the dome of a CFW/L1 mouse containing 51 unit segments, i.e. it represents a length of 561 μm of the follicle-associated epithelium.

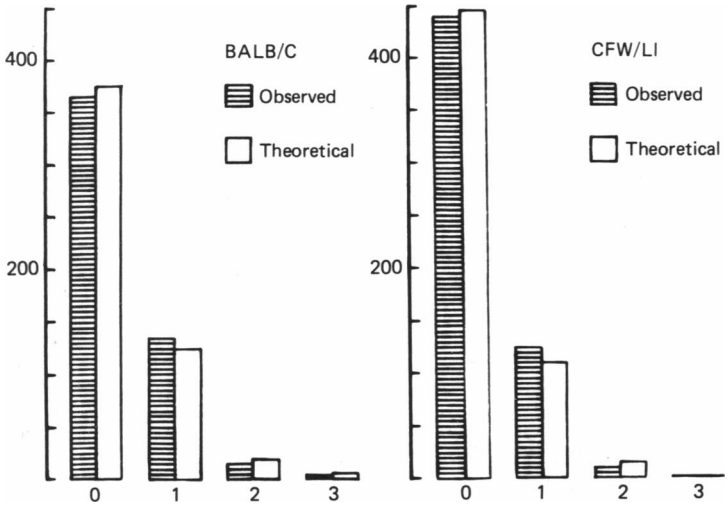

Fig. 5. The observed frequency distribution of the number of lymphoid cells per unit segment of the villus epithelium in the region of Peyer's patches (hatched histograms). The theoretical Poisson distributions, fitted to the observed means, are presented as white bars on the histograms. The observed (hatched) and theoretical (white) distributions are not significantly different for Balb/C and CFW/L1 mice. Vertical scale: frequency (number of segments); horizontal scale: number of lymphoid cells per segment.

D.F. = 2 for Balb/C mice, and $0{\cdot}25 > P > 0{\cdot}1$, D.F. = 1 for CFW/L1 mice. The close resemblance of the observed to the theoretical Poisson distributions (Fig. 5) supports the conclusion that the distribution of lymphoid cells per segment of villous epithelium associated with Peyer's patches is created by a random process in both strains of mice.

The pattern of spatial arrangement of segments containing lymphoid cells with respect to segments with no lymphocytes in villus epithelium was assessed by performing the test of randomness for runs. Theoretically, the spatial distribution of the segments containing lymphoid cells may be one of three possible patterns: even, random or non-random.

The number of runs in the villus epithelium of Balb/C mice was 212 which did not differ significantly from the theoretical value of 221 ($0{\cdot}5 > P > 0{\cdot}25$). In CFW/L1 mice the observed number of runs was 244 and the theoretical one was 228; the difference again was not significant ($0{\cdot}1 > P > 0{\cdot}05$). Therefore, segments containing lymphoid cells were interspersed randomly within the villus epithelium.

DISCUSSION

There are a number of morphological descriptions of the intraepithelial lymphoid cells in the region of Peyer's patches in mice (Chin & Hudson, 1971; Lemmel & Fichtelius, 1971; Abe & Ito, 1977, 1978; Owen, 1977; Bhalla & Owen, 1982) and in other species (Faulk *et al.* 1970; Bockman & Cooper, 1973; Owen & Jones, 1974; Chu *et al.* 1979; Burns, 1982), but there is no quantitative study of the spatial distribution of lymphoid cells along the epithelium.

The results revealed a new characteristic feature of the dome epithelium, namely

the non-random pattern of the intraepithelial lymphoid cells distribution. This has two independent aspects. Firstly, the distribution of lymphoid cells per segment (equal to the average distance between two neighbouring epithelial cell nuclei) of dome epithelium differs from that expected from the Poisson (random) distribution. This indicates the occurrence of segments of epithelium which are relatively rich in, and those depleted of, lymphoid cells. Secondly, segments in which lymphoid cells are either abundant or scanty occur in non-random clusters. Such clustering of lymphoid cells has been suggested but not proven, by Faulk *et al.* (1970) in rabbits and by Chu *et al.* (1979) in swine.

In contrast to the dome epithelium, the villus epithelium is characterised by a random (Poisson) distribution of the number of lymphoid cells per segment. The segments containing lymphoid cells are also distributed along the villus epithelium in a random manner, as proved by the test of randomness for runs. Earlier statements to this effect (Darlington & Rogers, 1966) have been based on visual impressions only.

The relative abundance of lymphoid cells within the dome epithelium (Faulk *et al.* 1970; Ferguson & Parrott, 1972; Bockman & Cooper, 1973; Owen & Jones, 1974; Abe & Ito, 1977, 1978; Chu *et al.* 1979; Smith *et al.* 1980) can be expressed as a ratio of the number of cells within a unit of dome epithelium to the number of cells within a unit of villus epithelium. The estimated ratio for Balb/C and CFW/L1 mice is about 3 and 4, respectively; the value given by Abe & Ito (1977) for dd strain of mice is about 2.

The reason for the higher frequency of lymphoid cells within the dome epithelium, as compared to the villus epithelium is not known. It may simply reflect the fact that a Peyer's patch dome is densely populated by lymphoid cells just beneath the epithelium, while the stroma of intestinal villi is not as densely populated by lymphocytes. However, this simple answer does not explain why crypts associated with Peyer's patch follicles, although close to aggregations of lymphoid cells in the stroma, do not contain intraepithelial lymphocytes, as shown in mice by Smith *et al.* (1980).

Therefore, other explanations should be sought. One possibility is that the follicle-associated epithelium of Peyer's patches lies upon a basement membrane of a special structure. This structure, different from that of the basement membrane of villus and crypt epithelium, would permit easier penetration of lymphoid cells into the epithelium of the dome. Possible differences in permeability of blood vessel walls to lymphoid cells in the two sites should also be taken into consideration.

Similar mechanisms may explain the occurrence of non-random clusters of lymphoid cells within the dome epithelium. In this instance, the barrier between the epithelium and the underlying lymphoid tissue should have sites which allow easy lymphocyte migration and other sites which do not. Burns (1982) claims that the basal lamina of dome epithelium in the fowl is discontinuous, as revealed by light microscopy of sections stained with van Gieson and the periodic acid–Schiff techniques. On the other hand, published electron micrographs of the dome epithelium (Owen, 1977; Bhalla & Owen, 1982) show the basal lamina (in the sense of the adepithelial lamina of electron microscopists) to be continuous. Ultrastructural evidence is of limited significance here due to the extremely small samples represented by electron micrographs.

It is well known that the dome epithelium contains a specific cell type, the M cell, associated with the lymphocytes (Owen & Jones, 1974; Owen, 1977; Chu *et al.* 1979;

Smith & Peacock, 1980). Is there, then, a causal relationship between the occurrence of M cells and the clusters of intraepithelial lymphoid cells? One may think of M cells as those cells which attract and/or retain lymphocytes. Conversely, lymphocytes migrating into the dome epithelium may bring about a differentiation of the epithelial absorptive cells into M cells, as proposed by Smith & Peacock (1982).

In the present study M cells could not be identified due to the limited resolution of the light microscope. Therefore, it is not possible to say that local accumulations of intraepithelial lymphoid cells coincide with the presence of M cells, although this seems highly probable because of the frequent occurrence of several lymphocytes associated with one M cell as described in man (Owen & Jones, 1974), mice (Owen, 1977) and in swine (Chu *et al.* 1979). The only quantitative study of the distribution of M cells in mouse dome epithelium (Smith & Peacock, 1980) gives no indication of the randomness or non-randomness of their distribution.

The role of lymphatic tissue associated with the digestive tract in the defence mechanisms of an organism has been disputed for a century, and the specific functions of lymphoid cells within the epithelia of the digestive tract remain a matter of hypothesis and speculation (Ferguson, 1977). It is postulated that the close association of lymphoid cells and dome epithelium is necessary for the recognition of antigens contained in the intestinal lumen by the lymphocytes (Ferguson, 1977).

The immunological significance of the non-random clusters of lymphoid cells within the dome epithelium, demonstrated in this paper, is not known. However, the local accumulation of lymphoid cells in the epithelium might be brought about by immunological reaction products within the epithelium. For example, substances similar to lymphokines could inhibit the migration of lymphoid cells and/or cause a local increase in permeability of capillaries. This last hypothesis is corroborated by the results of Ferguson & Parrott (1972) which show that the presence of intestinal luminal antigens is necessary for the infiltration of intestinal epithelium by lymphocytes.

The anatomical phenomenon of clustering of lymphoid cells within the follicle-associated epithelium, previously suggested and now established quantitatively by objective histometric and statistical procedures, awaits further studies of its biological significance.

SUMMARY

The spatial distribution of intraepithelial lymphoid cells in follicle-associated and villus epithelium within Peyer's patches was studied quantitatively in Balb/C and CFW/L1 mice. The results were essentially the same for both strains.

The distribution of the number of lymphoid cells per segmental length (equal to an average width of an epithelial cell) of dome epithelium was found to be non-random, since it deviated significantly from the theoretical Poisson distribution. Some segments of the dome epithelium, therefore, are lymphocyte-rich and some are lymphocyte-depleted, i.e. they contain either more, or less lymphoid cells than expected from the random distribution.

This was in contrast to the distribution in the epithelial layer of the villi, in which the distribution of the number of lymphoid cells per segment followed the Poisson distribution.

It was also demonstrated that segments rich in lymphocytes in the dome epithelium occurred in series or clusters. Lymphoid cell clustering was not observed in the villus epithelium.

Possible mechanisms responsible for the non-random spatial distribution of lymphoid cells within the dome epithelium of the mouse Peyer's patches are discussed.

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