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THE EFFECT OF ORAL IMMUNIZATION ON THE POPULATION OF LYMPHOCYTES MIGRATING TO THE MAMMARY GLAND OF THE SOW

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ABSTRACT

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Sows were immunized orally with live *Escherichia coli* according to various immunization schedules. Six pregnant gilts were used; 4 immunized at various intervals during the last month of gestation, 1 control immunized after parturition following suppression of lactation by weaning and 1 non-immunized control. The effect of oral vaccination on cell populations from lymphoid organs was studied. The in vitro proliferative responses of the cell populations to K88 antigen, anti-Ig sera and mitogens were used to demonstrate the distribution of sensitized lymphocytes over different lymphoid organs. The capacity of these cells to produce antigen-specific Ig was determined by in ovo translation of their mRNA.

Oral administration of antigen resulted in the appearance of K88-positive cells in lymphoid organs. In lactating sows, sensitized cells preferentially occurred in the mammary lymph nodes, whereas after suppression of lactation such a distribution was not seen. A possible route of migration of sensitized lymphocytes is discussed in relation to the local immune response. The antibody isotype produced by sensitized lymphocytes seemed to depend on the immunization schedule. The most effective schedule was one starting early in gestation and comprising frequent administration of antigen. This caused an optimal distribution of sensitized lymphocytes capable of IgA production.

INTRODUCTION

In view of the fact that in swine no transplacental transport of immunoglobulins occurs, colostrum and milk are the only sources of antibodies for newborn piglets (Bourne, 1976; Kortbeek-Jacobs and van der Donk, 1978, 1981). Protection against neonatal infections is solely provided by maternal

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antibodies. Knowledge of the mechanisms(s) by which the antibodies resulting from immunization enter the mammary secretions may contribute to the prevention of neonatal infectious diseases. We have already reported, that in sows' colostrum and milk, cells are present that contain mRNA coding for antibodies directed against intestinal antigens (Kortbeek-Jacobs and van der Donk, 1981). These committed cells, capable of producing Ag-specific immunoglobulins, are presumed to originate from the gut.

Administration of antigen by the oral route provokes sensitization of lymphocytes in the gut, and these cells may selectively migrate (i.e., home) to the mammary gland (Goldblum et al., 1975; Weiss-Carrington et al., 1977). Homing of IgA-containing cells has been studied by adoptive transfer of cells derived from several lymphoid tissues in rabbits (Craig and Cebra, 1971) and in mice (McWilliams et al., 1977; Roux et al., 1977). These experiments provided evidence for a common mucosal system which includes the intestines and the mammary gland (Bienenstock et al., 1978).

Lymphocyte stimulation tests were carried out on cells isolated from organs through which the lymphocytes might pass on their way to the mammary gland. The lymphocyte proliferative response to antigen and anti-isotype sera provides information about the distribution of sensitized cells over these organs. In addition, we performed in ovo translation experiments with the same cell populations (Kortbeek-Jacobs and van der Donk, 1978). This method provides supplementary information about the capacity of these cells to produce immunoglobulins specific for the administered antigen.

This paper describes experiments designed to determine the extent and the degree of lymphocyte proliferative response following oral exposure of pregnant gilts to *Escherichia coli* and to define the best method of stimulating production of antibodies by the mammary gland, in order to protect newborn piglets against this infection.

MATERIALS AND METHODS

Animals

The Dutch Landrace gilts used in the experiments were raised at the breeding farm of the Dutch Central Veterinary Institute and transferred to our stables at the beginning of the immunization procedure.

Four sows were immunized orally with doses of 200 ml of an overnight tryptone soya broth-culture of *E. coli* 08:K87:K88ab (0.8×10^9 viable organisms per ml). The doses were given in feed on 3 consecutive days per week, according to the schedules illustrated in Table I. One sow was used per schedule. The final dose was given at the onset of lactation. Six pregnant gilts were used; 4 immunized orally at various intervals during the last month of gestation, 1 control immunized after parturition following suppression of lactation by weaning, and 1 control which was not immunized.

The sows were killed 5 days after the last antigen dose was given and the

TABLE I

Immunization schedules used in the present study, 1 sow was used for each schedule; + = antigen administered for 3 consecutive days

Schedule	Days before parturition				
	28	21	14	7	0
I	–	–	–	–	–
II	–	–	–	+	+
III	–	–	+	–	+
IV	+	–	–	–	+
V	+	+	+	+	+

following lymphoid organs removed aseptically: Peyer's patches (PP); mesenteric lymph nodes (MLN); mammary lymph nodes (MaLN); external subiliac and prescapular lymph nodes (peripheral lymph nodes, PLN); spleen. Heparinized blood samples (PBL) were taken before slaughter.

Antisera and K88 antigen

Antisera directed against IgA, IgG and IgM (Jacobs et al., 1977) were raised in the goat. IgG fractions of goat antisera were prepared by precipitation with ammonium sulphate and ion-exchange chromatography. The same procedure was used to prepare IgG from non-immune goat serum as a control. Each antiserum was made specific for Ig heavy chains by affinity chromatography; the protein content was derived spectrophotometrically (E280 mμ = 13.8). The strain used for the preparation of K88 antigen was *E. coli* K12, C600:K88ab, a K12 strain conjugated with the wild type plasmid pRI8801. K88ab was isolated and purified according to the method described by Mooi and de Graaf (1979).

Lymphocyte proliferative response

The development of sensitized lymphocytes after immunization was studied by lymphocyte stimulation tests (LST) (van Dam et al., 1978; Shimizu and Shimizu, 1979). Preliminary studies with non-pregnant gilts were undertaken to standardize and optimize cell preparations and LST in swine. T-cell reactivity was studied by mitogenic stimulation with Con A and PHA, both strong mitogens for T-lymphocytes in swine (Outterridge et al., 1980). Anti-Ig sera were used to measure B-cell reactivity (Symons et al., 1977).

Lymphocyte preparation

Lymphoid organs (PP, MLN, MaLN, PLN and spleen) were cut into small pieces under sterile conditions. Cells were suspended in phosphate buffered

saline containing 0.2 g EDTA l^{-1} (PBS—EDTA) by gently squeezing the tissues. Peyer's patches were scraped with a scalpel to remove the upper mucus layer. The cell suspensions were then washed 3 times in PBS—EDTA by centrifugation for 10 min at 200 *g*, and finally resuspended using a 0.8 mm syringe, in RPMI-1640 medium containing 0.2% penicillin (100 U ml^{-1}) and 0.2% streptomycin (0.1 mg ml^{-1}). Cell preparations and blood samples (diluted 1:1 with RPMI-1640 medium) were layered onto an equal volume of Ficoll-Isopaque (d = 1.078 g cm^{-3}). Lymphocytes were separated by gradient centrifugation for 30 min at 450 *g*. Cells at the interphase were collected and washed twice with medium.

Lymphocyte stimulation tests in vitro

Cells were cultured in RPMI-1640 medium containing 10% newborn piglet serum, 1% 0.2 M L-glutamine, penicillin and streptomycin. Cultures of 200 μl, containing 2×10^5 cells, were incubated for 3 days at 37°C in a humidified atmosphere of 5% CO_2 in air. Eighteen hours before harvesting, the cells were labeled with ^{3}H-thymidine (0.4 μCi/culture; spec.act. 1.0 Ci $mmole^{-1}$). The cells were harvested with a multiple cell culture harvester (Cryoson) on to glass fibre filters. Radioactivity was counted in a Nuclear Chicago Liquid Scintillation Counter (efficiency 40—50%).

The lymphocyte proliferative response to K88 antigen was measured in cultures containing 5 μg purified antigen. The response to class-specific antisera was measured at concentrations of 8, 15, and 33 μg/culture of anti-α, -μ and -γ, respectively. The mitogens Con A and PHA were used in a concentration of 5 μg/culture. The results of the assays were expressed as the stimulation index calculated as follows:

$$SI = \frac{\text{mean cpm of triplicate stimulated cultures}}{\text{mean cpm of triplicate control cultures}}$$

In ovo translation of mRNA

In parallel with the LST experiments, lymphocytes from mammary and peripheral lymph nodes were cultured in the presence of K88 antigen, but without ^{3}H-TdR. These cells were injected into oocytes of *Xenopus laevis* according to the method previously reported (Kortbeek-Jacobs and van der Donk, 1978), with minor adaptions due to the use of oocytes instead of egg cells. Briefly, 50 of the cell suspensions (2×10^6 cells ml^{-1}) were injected into each of 40 oocytes. The injected oocytes were suspended in 1 ml of incubation medium (Gurdon et al., 1971) and incubated for 40 h at 17°C in the presence of ^{3}H-leucine (1 mCi ml^{-1}, spec.act. 140 Ci $mmole^{-1}$). After incubation, the oocytes were washed free of ^{3}H-leucine, homogenized and centrifuged for 30 min at 30 000 *g* at 4°C. The clear supernatants were collected for analysis by radioimmunoassays (RIA). In parallel assays for IgA, IgG,

IgM and the total amount of K88 antibodies, translated in ovo, were conducted as described by Kortbeek-Jacobs and van der Donk (1981).

RESULTS

Mitogens

In vitro responses to PHA and Con A of various lymphocyte populations from sows are shown in Table II. Lymphocytes from PLN, MLN and MaLN of immunized sows were more reactive than comparable lymphocytes from non-immunized animals. Less reactivity was found with lymphocytes from PBL and spleen, whereas lymphocytes from PP of both immunized and non-immunized animals showed the same reactivity.

TABLE II

Lymphocyte proliferative response of sows to T-mitogens. Lymphocytes were isolated from pregnant sows orally immunized with live *E. coli*. PBL = peripheral blood lymphocytes; PLN = peripheral lymph nodes; PP = Peyer's patches; MLN = mesenteric lymph nodes; MaLN = mammary lymph nodes

	Non-immunized		Immunized	
	PHA	Con A	PHA	Con A
PBL	392± 73[a]	346±103	123± 36	75± 17
PLN	884±598	585±208	1217±430	1289±555
PP	237	275	259±134	255±151
MLN	473±153	295± 58	1187±303	723±267
Spleen	305± 86	315±106	63± 7	43± 6
MaLN	460±322	141± 55	706±224	859±342

[a]Stimulation index (SI) = $\frac{\text{mean cpm triplicate stimulated cultures}}{\text{mean cpm triplicate control cultures}}$; mean ± SD, n=4.

K88 and antisera

The lymphocyte proliferative responses to K88 antigen are shown in Table III. Immunization resulted in the appearance of K88 positive cells. In lactating sows they occurred preferentially in MaLN, whereas in the controls such a distribution was not seen.

Table IV illustrates the response of lymphocytes from the same animals as given in Table III to class-specific antisera. The effect of immunization was to increase the SI in response to Ig-class-specific antisera. This may indicate a specific increase of cells bearing surface immunoglobulins (sIg-positive cells) of a certain class. In particular, an increase of sIgM-positive cells was de-

TABLE III

Lymphocyte proliferative responses of sows to K88 antigen of *E. coli*. Lymphocytes were isolated from pregnant sows orally immunized with live *E. coli*. PBL = peripheral blood lymphocytes; PLN = peripheral lymph nodes; PP = Peyer's patches, MLN = mesenteric lymph nodes; MaLN = mammary lymph nodes

	Non-immunized lactating K88	Immunized lactating K88	Immunized non-lactating[a] K88
PBL	2.7[b]	2.4± 0.4[c]	4.6
PLN	1.3	6.9± 1.4	7.0
PP	n.d.	2.1± 0.2	2.4
MLN	2.2	6.2± 1.9	4.0
Spleen	2.9	1	1
MaLN	1.5	10.6± 2.8	7.9

[a]Two weeks after parturition.
[b]Stimulation index.
[c]Mean ± SD, n=4.

TABLE IV

Lymphocyte proliferative responses of sows to Ig-class specific antisera. Lymphocytes were isolated from pregnant sows orally immunized with live *E. coli*. α = anti-IgA heavy chain; μ = anti-IgM h.c.; γ = anti-IgG h.c.; PBL = peripheral blood lymphocytes; PLN = peripheral lymph nodes; PP = Peyer's patches; MLN = mesenteric lymph nodes; MaLN = mammary lymph nodes

	Non-immunized lactating			Immunized lactating			Immunized non-lactating[a]		
	α	μ	γ	α	μ	γ	α	μ	γ
PBL	1.5[b]	12.0	1.6	2.1± 0.7[c]	2.2± 0.4	4.0± 1.3	1	2.0	5.5
PLN	1	3.1	1	2.0± 0.3	11.7± 4.3	2.8± 0.9	4.2	65	18
PP	n.d.	n.d.	n.d.	3.8± 0.1	3.4± 1.3	1.7± 0.1	1.2	2.2	1.0
MLN	1	10.0	3.8	7.4± 2.8	32.3± 1.4	8.7± 1.9	1.9	26	3.5
Spleen	1	1.1	1	1	1	1	1	1.2	1
MaLN	1	4.5	1	1.7± 0.4	54.3± 32	8.9± 4.3	1.0	25	3.4

[a]Two weeks after parturition.
[b]Stimulation index.
[c]Mean ± SD; n=4.

tected. These positive cells were found preferentially in PLN of non-lactating sows and in MaLN of lactating sows. The increase of sIgA-positive cells was most obvious in PP and MLN of lactating sows and in PLN (and MaLN) of non-lactating sows.

Although 4 different immunization schedules were used, only the mean stimulation indices are given in Tables II, III and IV. However, the most

striking result, i.e., the maximal reactivity detected for MaLN cells of lactating sows, was valid for all schedules. In Table V the results of the individual schedules are summarized and a comparison is made between the reactivity of lymphocytes of MaLN and PLN.

TABLE V

Lymphocyte populations in mammary and peripheral lymph nodes after different immunization schedules compared to a control. Lymphocytes were characterized by their in vitro proliferative response to K88 antigen (SI) and by their capacity to synthesize specific antibodies (cpm). All sows were lactating at the time of the tests. For details concerning the immunization schedules refer to Table I.

Immunization schedule	Mammary lymph nodes				Peripheral lymph nodes			
	SI[a]	Anti-K88 (cpm)	Ig (cpm)	Anti-K88/ Ig	SI	Anti-K88 (cpm)	Ig (cpm)	Anti-K88/ Ig
I	1.5	2292	11695	0.20	1.3	3511	10105	0.35
II	3.7	3080	4126	0.75	9.3	15611	40277	0.39
III	7.5	n.d.	n.d.	—	4.3	n.d.	n.d.	—
IV	12.0	6980	21256	0.33	4.1	4958	21866	0.23
V	19.0	n.d.	n.d.	—	9.9	n.d.	n.d.	—

[a] SI = stimulation index, defined by $\frac{\text{mean cpm of triplicate stimulated cultures}}{\text{mean cpm of triplicate control cultures}}$;

n.d. = not done.

In ovo translation of mRNA

In addition to their proliferative response to K88 antigen, the cell populations of MaLN and PLN were studied with respect to their mRNA coding for K88 antibodies and their mRNA coding for immunoglobulins. The results are shown in Table V.

Lymphocytes of MaLN and PLN showed a positive correlation between proliferative response to K88 antigen indicated by the stimulation index, and in ovo synthesis of K88 antibodies, determined by incorporation of radioactivity, anti-K88 (cpm). The proportion of anti-K88 to that of total translated Ig (anti-K88/Ig) increased by oral immunization in MaLN and was unchanged or even decreased in PLN, compared to the corresponding control.

The cell populations were analyzed further with respect to the isotypes that they may synthesize. Each of the lymphocyte populations was split into 2 cultures. One culture was stimulated in vitro with K88 antigen and the other was not. The in vitro stimulation can be expected to cause proliferation of the antigen-specific cells. As a result, more antigen-specific cells and thus more specific mRNA should be present in stimulated cultures than in non-stimulated cultures. Therefore, by comparing the mRNA-content of

stimulated and non-stimulated cultures, conclusions can be drawn with respect to the isotype(s) involved in the response to K88. The results of these experiments are summarized in Fig. 1. In the cells cultured in the presence of K88 antigen, the proportion of IgA-, IgG- and IgM-mRNA in the total Ig-mRNA in the cell populations of mammary lymph nodes had changed.

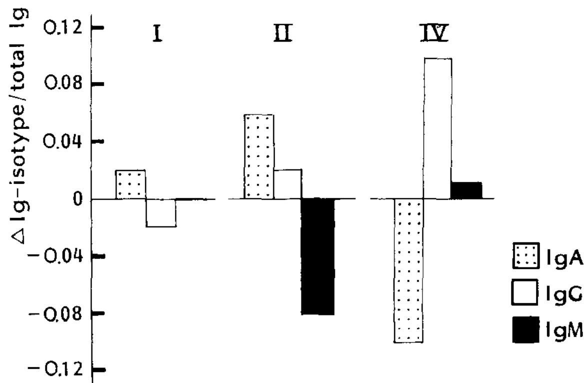

Fig. 1. Isotype expression by cells from mammary lymph nodes as a result of in vitro stimulation with K88 antigen. The difference between cells cultured with and without K88 antigen is expressed as the proportion of each Ig-class to that of total Ig. I = control; II, IV = sows immunized according to schedules II and IV, respectively (see Table I).

As the control sows were negative with respect to serum antibodies to K88 (detectable by ELISA) and in vitro proliferative response to K88 antigen (Table V, Schedule I), the increase of IgA-mRNA in the control lymphocyte populations reflects the primary reaction of lymphocytes in vitro to the antigen. However, since the proliferative response to antigen was low, only a small number of cells reacted in vitro. After the short immunization schedule (II), a positive IgA shift was found in the mammary lymph nodes. This indicated an increased number of cells, which were committed to IgA-synthesis due to antigen stimulation. With Schedule IV immunization was started earlier in gestation and as a result of a booster application at the onset of lactation, many of the lymphocytes in the mammary lymph nodes contained mRNA coding for IgG.

DISCUSSION

This study was done using a small number of animals and we are aware of the fact that conclusions are rather speculative. However, the results have been evaluated in the best possible way to show the effect of oral vaccination on lymphocyte populations of the sow. In a previous paper (Kortbeek-Jacobs and van der Donk, 1981), we reported data concerning Ig-production in mammary secretions as a result of oral immunization. The experiments described in this paper were carried out to complement earlier findings.

Distribution of sensitized lymphocytes

The various immunization schedules resulted in comparable in vitro responses to K88 antigen, T-mitogens and anti-Ig. Some variation was observed in the lymphocyte populations of MaLN, MLN and PLN. The overall effect of oral immunization of pregnant sows was an increase of K88-positive cells in lymph nodes, but not in the spleen. The distribution of these cells was related to lactation, so that in lactating sows sensitized lymphocytes were found predominantly in the mammary lymph nodes, whereas in non-lactating sows the response to K88 antigen was equally high in the mammary and peripheral lymph node lymphocytes. The distribution was essentially the same after each immunization schedule, therefore, the results from the various groups have been pooled and data are presented as the mean SI.

A closer analysis of the data (Table V, SI) reveals that after the short immunization Schedule II, which started 7 days before parturition, PLN lymphocytes were more reactive to K88 antigen than MaLN lymphocytes. This indicates that the time between first antigen administration and parturition has to be more than 1 week. Chidlow and Porter (1979) recommend 18 days for the booster application in their immunization procedure, which was a combination of oral priming and parenteral boosting. In designing an immunization schedule, the development of the mammary gland in the final period of gestation must be taken into account.

Route of migration

Evaluation of our results indicates that there is a specific route of migration, which is shown in Fig. 2. Antigenic stimulation takes place in the small

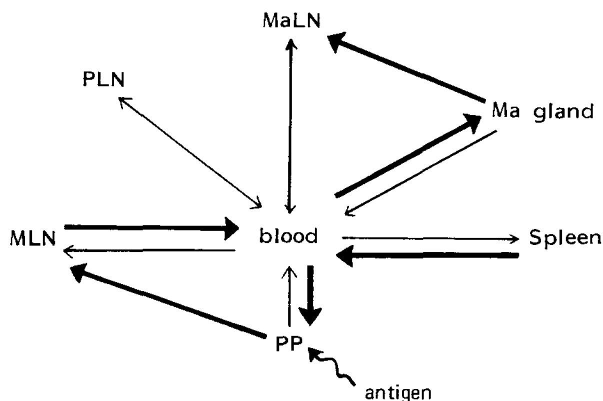

Fig. 2. Suggested routes of migration of antigen-sensitive lymphocytes in the lactating sow. After sensitization in the gut, lymphocytes migrate via MLN and blood to the mammary gland. Draining to MaLN occurs with afferent lymph. PP = Peyer's patches; MLN = mesenteric lymph nodes; PLN = peripheral lymph nodes; MaLN = mammary lymph nodes; Ma gland = mammary gland.

intestines at the site of Peyer's patches. According to Binns (1973, 1980), lymphocyte migration patterns in swine are not concordant with those in other species: lymphocytes enter the lymph nodes via the blood as in all mammalian species, but they also return to the blood directly from the node. This implies that in swine, lymphocytes travel mainly via the bloodstream.

In view of our data concerning the lymphocyte population in MaLN, we propose that lymphocytes migrate with lymph from the root area in the mammary gland to the regional node. This occurs during lactation, when the mammary gland is filled with fluid and contains numerous sensitized lymphocytes. Oral administration of antigen causes traffic of sensitized lymphocytes from PP via MLN and blood, to MaLN and/or PLN, depending on lactation.

We did not find any stimulation by K88 antigen of lymphocytes in the spleen of immunized sows and their response to mitogens was markedly decreased (Table II). Therefore we suggest, that the lymphocyte population of the spleen of swine comprises small lymphocytes which can be recruited by oral immunization; this is marked by a decreased reactivity to mitogens following antigen application.

After sensitization, lymphocytes migrate to PLN or MaLN but not the spleen. Immunohistochemical studies (Brown and Bourne, 1976; Jacobs and Goudswaard, 1977) have already demonstrated that the number of mature plasma cells in the spleen is very low. In a recent publication, Pabst and Nowara (1982) demonstrated that spleen-derived small lymphocytes migrate to mucosal sites such as the lung, tonsils and Peyer's patches. This confirms our opinion, that the spleen plays a role in mucosal immunity as a source of lymphocytes. Once the antigen enters the intestines more spleen lymphocytes are recruited, which migrate to the gut where they are stimulated by antigen and then migrate to mucosal sites.

Productive capacity

The second parameter used to study the effectiveness of immunization was the capacity of sensitized lymphocytes to produce Ig and especially secretory Ig. In man and several animal species (Tomasi and Bienenstock, 1968; Goldblum et al., 1975), IgA is the predominant Ig in the mucosal immune system. In the young piglet, IgM also plays an important role in the defense against neonatal infections (Chidlow and Porter, 1979).

The correlation between K88 and IgM-positive cells, which migrate to the same lymphoid organs as was found by in vitro stimulation tests, seems to confirm these findings (Tables III and IV). However, by LST-only cells bearing surface Ig were detected. This implies that plasma cells which actually produce Ig are not found by this method. The proportion of IgA^+ and IgM^+ cells does not reflect the proportion of the respective plasma cells.

In the MaLN, predominantly IgM-positive cells were detected, whereas

IgA-positive cells predominated in PP and MLN. These IgA^{+} cells are presumed to be committed lymphocytes, that differentiate further into IgA-producing plasma cells once they have reached the mammary gland. A very good tool in the measurement of the productive capacity of cells is in ovo translation of mRNA (Kortbeek-Jacobs and van der Donk, 1978). By applying this method to the same isolated cell populations used for LST, we obtained data on Ig production after the different immunization schedules (Table V, Fig. 1).

IgA was expressed after the short Schedule II and IgG after Schedule IV, which started early in gestation (Fig. 1). Although MaLN were rich in antigen-specific cells after Schedule IV, they expressed IgG, which may be of less importance in the local protection of neonatal piglets. Previous experiments have already indicated a prevalence of cells containing IgA- and IgM-mRNA, specific for K88 antigen, in the colostrum of sows orally immunized according to Schedule V (Kortbeek-Jacobs and van der Donk, 1981). The experiments reported here complement these findings. They prove that frequent administration of the antigen for long periods leads to an optimal localization in the mammary gland of antigen-sensitive cells capable of Ig production.

Schedules IV and V differed in the frequency with which antigen was administered. As a result, anti-K88 activity was found to be in the IgG (this paper) and IgA classes. In vitro stimulation of lymphocytes from sows which had not been immunized suggested that the response to K88 antigen in the IgA class could be attributed to priming of lymphocytes.

Therefore, we conclude that in swine, surface IgA-bearing cells are activated as a result of the primary response to K88 antigen. The effect of Schedule V probably can be explained as a continuous priming of lymphocytes.

Our results indicate that the migration of cells, committed to Ig synthesis, to the mammary lymph nodes depends neither on the immunoglobulin class that is expressed by the lymphocytes (Fig. 1) nor on the presence of specific antigen (Kortbeek-Jacobs and van der Donk, 1981). Most probably, it is coinciding with events under hormonal control such as the development of mammary gland epithelium, as was demonstrated in mice by Weiss-Carrington et al. (1977). When lymphocyte distribution and productive capacity are considered, immunization Schedule V fits best in the phenomenon of homing occurring in the pregnant animal. This schedule results in an optimal immunity for the sow which is consequently most profitable for her offspring.

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