Agents and Actions, vol. 32, 3/4 (1991)

0065-4299/91/040160-07 $ 1.50 + 0.20/0

The anti-inflammatory activity of a low molecular weight component derived from the milk of hyperimmunized cows

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Abstract

"Immune" milk has been utilized as a source of biologically active compounds for many years. In the present study, a low molecular weight fraction, isolated from the milk of dairy cows hyperimmunized with a multivalent bacterial vaccine (HIMF), has been evaluated for anti-inflammatory activity. Analysis was carried out using the rat hind-paw oedema assay. HIMF was shown to have a marked anti-inflammatory effect in this model and carrageenin-induced oedema was suppressed by up to 80% in individual experiments. The agent was active following oral, subcutaneous, intramuscular, intraperitoneal or intravenous administration. Intravenous injection was particularly effective and amounts as small as 1 mg significantly reduced the inflammatory response to carrageenin. The experiments have established that milk from hyperimmunized cows contains a highly active anti-inflammatory compound and form a basis for further studies, which will attempt to isolate and further characterize the active moiety.

Introduction

The advantages of feeding breast milk to the new born have long been recognized, but it is only recently that its value has been considered in other than nutritional terms [1]. Breast milk components contribute to host protection by providing the infant with preformed antibody and interfering with the ability of pathogenic microorganisms to adhere to and invade tissues [2, 3]. Other constituents with anti-inflammatory activity limit tissue disruption in the gastrointestinal tract during infectious episodes and help maintain normal absorption and maturation [4]. The involvement of milk in host protection can also be inferred by the presence of IgA, complement, lysozyme and lactoferrin [5] and the recent demonstration that a fraction from the milk of immunized cows reduced the level of experimentally induced mastitis in mice [6]. These results and the pioneering work of Petersen and colleagues on the properties of milk from cows subject to various bacterial challenges [7], have stimulated efforts to isolate and characterize biologically active components of milk. In the present experiments, a low molecular weight fraction isolated from the milk of dairy cows, hyperimmunized with a multivalent bacterial vaccine, has been demonstrated to contain a remarkably active anti-inflammatory component.

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Materials and methods

Animals

Female Dark Agouti rats, approximately 12 weeks old, weighing between 160 and 180 g and obtained from an inbred colony, were used in these experiments.

Carrageenin-induced footpad oedema

Inflammatory oedema was induced by the subcutaneous injection of 0.1 ml of 2% kappa-carrageenin (Sigma type III) into the plantar region of each hind footpad [8]. All injections and measurements were carried out under light anaesthesia.

Measurement of oedema using ^{125}I labelled human serum albumin (^{125}I-HSA)

At the time of footpad challenge with carrageenin, each rat was injected intravenously via the ventral tail vein with 1 μCi of ^{125}I-HSA. Four hours later, 2 ml of blood was obtained by heart puncture from anaesthetized animals and transferred into a tube containing EDTA. The rats were then killed by the intracardiac injection of air. The hind feet were then removed at the tarsal joint with paediatric rib-cutters and placed in 100 × 17 mm polystyrene counting tubes. Blood was separated by centrifugation and 200 μl of plasma diluted in 800 μl of distilled water in a 75 × 11 mm polystyrene tube. The level of radioactivity in each foot and in the plasma samples was then determined using a Philips model 4580 automatic gamma counter. The volume of the foot is expressed in μl of plasma equivalents, calculated as follows:

$$\frac{\text{cpm/foot} - \text{background cpm}}{\text{cpm/μl plasma} - \text{background cpm}}$$

There is a direct relationship between the background plasma volume of the foot and the weight of the rat. Using a standard curve, constructed from this data the baseline plasma volume was calculated for each animal and subtracted from the 4 h volume following carrageenin challenge. Thus, the values reported represent the absolute oedema volume.

Hyperimmune milk fraction (HIMF)

This material is a freeze-dried fraction of bovine whey, prepared from milk obtained from dairy cows hyperimmunized with a multivalent killed bacterial vaccine and was a kind gift from Stolle Milk Biologics International, Cincinnati, Ohio. Primary immunization was carried out using 2×10^9 killed bacteria cells. Cows were injected at weekly intervals for four weeks beginning 2–3 days before parturition. Booster injections are given every two weeks to maintain the animals in a hyperimmunized state. Details of the preparation, composition and administration of the vaccine and the milk processing procedure, have been described [9]. The hyperimmune milk fraction was prepared from rennet whey by a proprietary sequence of ultrafiltration and size exclusion chromatography [10]. The carbohydrate content of the fraction is consistent with a polymeric or oligomeric material with some carbinol side chains that have been oxidized to carbonyl groups. The material contains traces of nitrogen and phosphorus and significant amounts of C18 fatty acids. The active component is contained in a matrix of lactose.

Control agents

a) Aspirin: each assay was validated by including a group treated with the known anti-inflammatory agent, aspirin. Soluble aspirin was dissolved in water and administered by gastric gavage at a dose of 200 mg/kg, 30 min before challenge.

b) Normal milk fraction: in order to control for counterirritancy and other non-specific effects, a lactose fraction was prepared from the milk of non-immunized cows and used as a control for the HIMF. This normal milk fraction is referred to as NMF.

c) Non-active milk fraction: the normal milk fraction described above was prepared from a randomly collected, commercial milk supply. In order to provide a definitive control fraction, a "twin herd" of 60 animals, consisting of 30 sets of identical twins was established at the New Zealand Dairy Board Research centre at Ruakura. One of each pair of twins was injected with the bacterial antigen according to the schedule described above and the milk from two herds was collected separately. The whey from each pool was then processed using the same fractionation procedures employed to prepare the HIMF. Thus the fraction from the non-immunized cows (TH-NMF) provided an appropriate control material with which the corresponding fraction from the milk of immunized cows (TH-HIMF) could be compared.

Experimental protocol

In individual experiments, between four and ten groups of rats, each comprising six animals, were

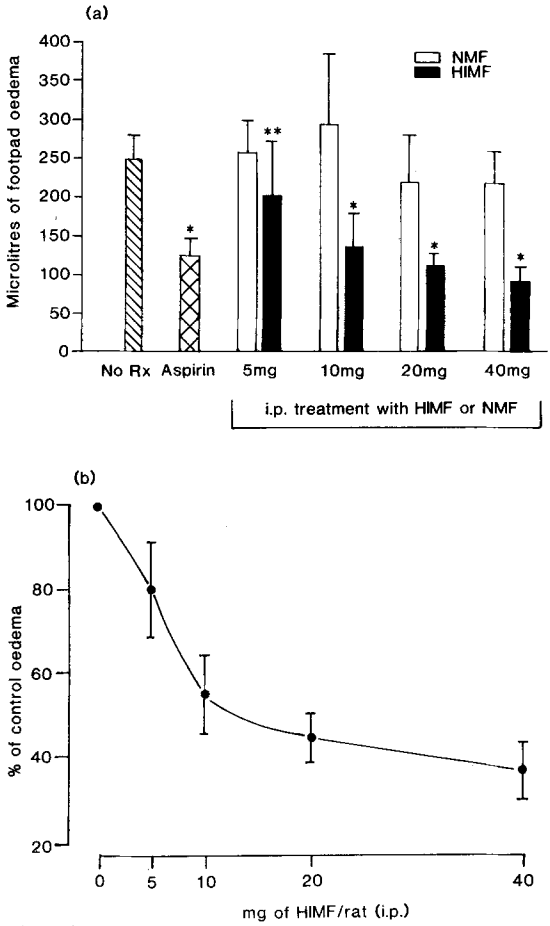

Figure 1
Effect of i.p. injection of hyperimmune milk fraction (HIMF) on carrageenin induced oedema of the rat hind paw. (**a**) Comparison of HIMF treated rats with non-manipulated controls, aspirin treated animals and animals treated with normal milk fraction (NMF). (**b**) Dose response of the anti-inflammatory effects of HIMF. The lower the percentage value, the greater the anti-inflammatory effect. $*=p<0.01$ and $**=p<0.02$ when HIMF treated animals are compared with NMF treated controls. The aspirin treated group were compared with the non-treated controls.

used. For each experiment, animals were selected which were uniform in terms of age and weight. Two control groups were employed for each experiment; the first was an untreated control group in which the only manipulation was the injection of carrageenin into the footpads. A second control group was treated with aspirin at a standard dose of 200 mg/rat to confirm that the carrageenin-induced oedema could be inhibited by a recognized anti-inflammatory agent. The other groups were treated with HIMF or NMF as detailed in the individual protocols for each experiment.

Statistical evaluation

Results were compared using the Wilcoxon sum of ranks test for non-parametric data. Error bars represent one standard deviation.

Results

Effect of intraperitoneal administration of HIMF on the carrageenin-induced oedema in the rat footpad

Sixty animals were divided into ten equal groups, comprising a non-treated control group, a group treated with 200 mg/kg of aspirin p.o., four groups injected i.p. with either 5, 10, 20 or 40 mg of HIMF and a further four groups given either 5, 10, 20 or 40 mg of normal milk fraction (NMF) i.p. The various treatments were administered 30 min before footpad challenge with carrageenin. In the untreated control group a mean volume of 250 µl of oedematous fluid accumulated in the carrageenin challenged footpad. Aspirin pre-treatment limited the mean oedema volume to 50% of this value (Fig. 1 a). Intraperitoneal administration of HIMF at all four doses (5, 10, 20 or 40 mg) resulted in a highly significant inhibition of oedema (to 80, 63, 56 and 45% of the control oedema respectively). When these mean percentage values were plotted against the quantity of HIMF administered, the dose-response curve shown in Fig. 1 b was obtained. NMF, also administered i.p., did not inhibit inflammation.

Comparative efficacy of HIMF administered by different routes

Sixty rats were divided into 10 equal groups. One group remained untreated and, as an assay control, a further group was given aspirin p.o. at 200 mg/kg. The remaining eight groups were treated with HIMF or NMF at 40 mg/rat using either the subcutaneous, oral, intramuscular or intravenous routes. The agents were administered according to

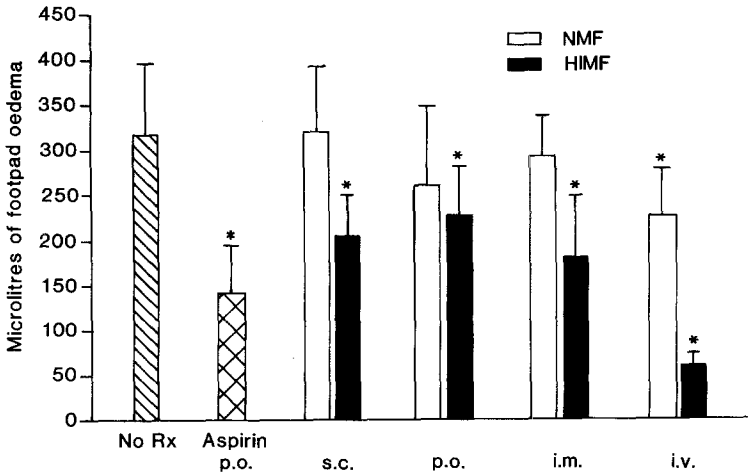

Figure 2
The relative anti-inflammatory activity of the hyperimmune milk fraction (HIMF) and normal milk fraction (NMF) when administered by the subcutaneous (s.c.), oral (p.o.), intramuscular (i.m.) and intravenous (i.v.) routes. HIMF or NMF was given at a dose of 40 mg/rat. $*=p<0.01$ when HIMF treated animals are compared with NMF treated controls. The aspirin treated group were compared with the non-treated controls.

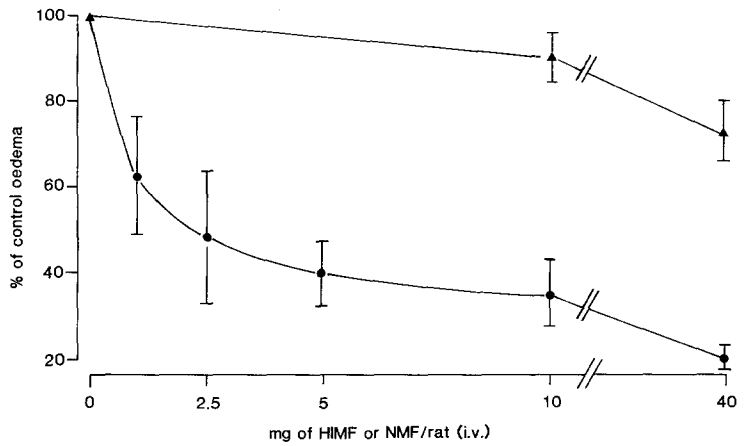

Figure 3
The effect of increasing intravenous doses of hyperimmune milk fraction (HIMF) and normal milk fraction (NMF) on the inhibition of footpad oedema. ●—● = HIMF, ▲—▲ = NMF.

the following schedules:

Agent	Route	Time before carrageenin challenge
Aspirin	p.o.	30 min
HIMF/NMF	s.c.	60 min
HIMF/NMF	p.o.	24 h, 16 h & 1 h
HIMF/NMF	i.m.	30 min
HIMF/NMF	i.v.	0 min

The results of the experiments are shown in Figure 2. All four modes of HIMF administration led to a significant inhibition of oedema. Subcutaneous, oral, and i.m. administration limited the oedema volume to 64, 72 and 57% of the oedema in the control animals respectively, while i.v. administration at this dose almost totally abrogated the response to carrageenin (19% of control oedema). NMF, given by the s.c., p.o. or i.m. routes, did not significantly inhibit the response to carrageenin, but a 40 mg intravenous dose did result in a small,

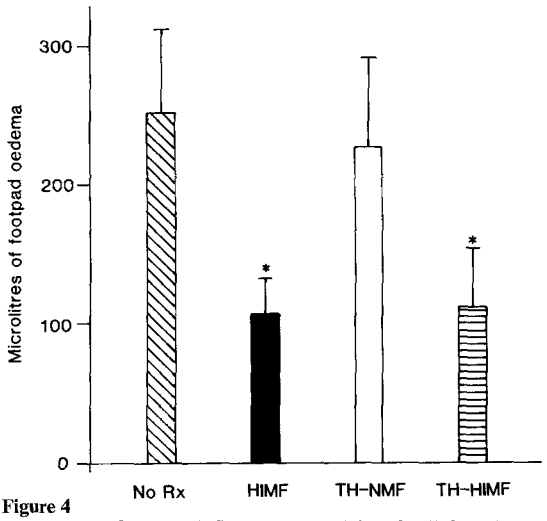

Figure 4
Verification of the anti-inflammatory activity of milk from hyperimmunized cows using a twin herd. The hyperimmune milk fraction (TH-HIMF) was prepared from pooled milk obtained from hyperimmunized cows while the normal milk fraction (TH-NMF) was obtained from the milk of their non-immunized twin sisters. Both preparations were injected i.v. at a dose of 4 mg/rat. * = $p < 0.01$ when compared with the non-treated control group.

but statistically significant inhibition of oedema volume (71% of control oedema).

Dose response to intravenous administration of HIMF

Nine groups of six animals were used. One group was untreated and served as a control, while a second was given aspirin as before. Five of the groups were given HIMF i.v. at doses of 1, 2.5, 5, 10 or 40 mg/rat while the remaining two groups were treated with NMF i.v. at either 10 or 40 mg/rat i.v. HIMF produced a dose related inhibition of the oedematous response to carrageenin (Fig. 3). NMF at 10 mg had no effect on the response. The 40 mg dose did however produce a significant decrease, but the effect was minimal compared to an equivalent amount of HIMF.

Verification of anti-inflammatory activity in the HIMF using a twin herd

Fractions were prepared from a pool of milk, obtained from either the immunized or non-immunized siblings of a twin herd (TH) of Jersey cows. The fraction from the immunized animals was designated TH-HIMF and that from the non-immunized animals TH-NMF. Twenty four rats (four groups of 6) were used in the analysis of these two compounds. One group of 6 animals was untreated and served as controls, each individual in the second group was injected with 4 mg of HIMF, animals in the third group were each given 4 mg of TH-HIMF, while all members of the final group were treated with 4 mg of the TH-NMF. All agents were injected i.v. at the time of footpad challenge. Administration of HIMF or TH-HIMF led to a highly significant inhibition of footpad oedema (to 42 and 44% of control oedema respectively). In contrast the identical fraction prepared from the milk of the control animals (TH-NMF), had no effect on the inflammatory response (Fig. 4).

Discussion

The present study was carried out to evaluate the anti-inflammatory activity of a low molecular weight milk fraction isolated from hyperimmune cows. The material was particularly active in the carrageenin paw oedema model of inflammation following intravenous administration, but was also shown to be anti-inflammatory when given by the subcutaneous, oral or intramuscular routes. Dose response curves were established and the compound was shown to be active at very low doses, without overt signs of toxicity.

The demonstration of an anti-inflammatory factor in the milk of hyperimmunized cows is the outcome of a series of observations and laboratory studies on the properties of "immune" milk initiated by Petersen and colleagues [7, 11] and continued by Stolle Milk Biologics, Cincinnati, Ohio [9, 10]. The important function of milk in the transfer of immune protection from a lactating mother to a nursing infant had been proven before the turn of the century. However, the role of milk in protection against infectious disease was largely forgotten after the discovery that infection could be treated using serum from immunized animals and, more recently, with antimicrobial agents. In 1955, Petersen and Campbell discussed evidence that the milk of cows, hyperimmunized via the teat canal with a mixture of killed *Streptococcus haemolyticus* and staphylococci, contained high levels of antibodies which offered protection against specific diseases in man when absorbed from the gastrointestinal tract [12]. Uncontrolled trials were later carried out by Smith, a Minnesota physician, who conducted a survey of the effect of

consuming immune milk on the symptoms of rheumatoid arthritis [13]. Of the 199 individuals studied, 57% claimed that their condition improved with the consumption of the milk. Rationale for the therapy was based on the concept that antibody absorbed from the immune milk neutralized corresponding antigens in plasma and prevented the formation of antigen-antibody complexes known to precipitate in the joints of such patients.

Investigations carried out by Stolle Milk Biologics were based on the principles espoused by Petersen, but using an alternative immunization schedule, involving intramuscular injection of a wider range of killed bacteria. Encouraging results from uncontrolled trials led to a double-blind placebo-controlled clinical study being carried out to evaluate the effect of immune milk on subjects with rheumatoid arthritis. It was claimed this trial demonstrated that the benefit of immune milk consumption was not due to a placebo effect or the nutritional benefits of a daily intake of milk [9].

Recent attempts to isolate biologically active components from the milk of hyperimmunized cows have taken into account the many advances that have occurred in understanding the biology of the immune and inflammatory responses. As a result, the original view that the effects of immune milk on inflammatory processes were mediated through specific antibody has largely been discarded. Current theory favours the concept that the vigorous immunization schedule leads to an increase in the synthesis and excretion in the milk, of mediators involved in the control of the immune and inflammatory responses. Milk is known to contain a number of components with anti-inflammatory properties and the concept has been advanced that, during an infectious episode in the respiratory or GI tract, these factors limit the tissue damaging inflammatory reaction which normally accompany the host response to infection [14]. Compared with an adult, the infant is much less able to tolerate disruption of normal gut or respiratory function and suppression of inflammation during infection confers considerable teleological advantage. The increase in the anti-inflammatory activity of milk from hyperimmunized cows could be related to this property, although there are clear differences in the composition of human and cows milk which need to be taken into consideration [15]. The question then arises as to how killed bacterial antigen, administered at a site well removed from mammary tissue, could lead to an increase in the level of anti-inflammatory activity in milk. Possibilities include the transfer of factors from plasma to milk, or local synthesis within the mammary tissue. The observation that the immunologic repertoire of the mammary gland reflects the infectious flora of the exposed mucosal surfaces and that most of the specifically reactive components contained in the lactation products are directed against organisms or antigens on the mucosal surface, supports the latter hypothesis. There is evidence too that plasma cells found in milk originate from the precursor immune-competent cells of the gut and bronchial lymphoid tissue and that exposure of these cells to microbial antigen at these sites is a prerequisite for their activation and proliferation. The antigen-sensitized cells are eventually transported via the systemic circulation to other sites, including the mammary glands, where they initiate the synthesis of immunoglobulin against the antigens previously encountered [16–20]. Studies have shown that bronchial immunization of rabbits with non-replicating antigens such as dinitrophenylated key hole limpet haemocyanin, generates specific primary and secondary responses in milk [21, 22] and that labelled thoracic duct lymphocytes from parasite infected rats accumulate in the mammary glands of lactating syngeneic hosts [23]. These findings support the proposal that the mammary gland functions as an extramucosal extension of the bronchial and intestinal lymphoid tissues. These studies have established that antigen deposition at a site remote from mammary tissue can lead to local synthesis and secretion of immunoregulatory factors in the milk. Although the immunoglobulins have been the molecules studied in greatest detail, the secretory repertoire of the lymphoreticular cells include a number of other biological response modifiers. These include the lymphokines [24–26], interferons [27], prostaglandins [28, 29] and complement components [30]. Other less well defined milk components with biological activity are also known to occur, such as a glycoprotein with immunosuppressive activity [31] and a heat stable factor with viral inhibitory properties [32, 33]. Chemical characterisation of the anti-inflammatory component of the hyperimmune milk is still at an early stage and there is no direct evidence associating any of the above with the anti-inflammatory properties of

HIMF. However, they do serve to illustrate the potential of hyperimmunization to affect the composition of milk and generate immunoregulatory agents capable of influencing the inflammatory response. Given the paucity of injectable non-steroidal anti-inflammatory drugs available to the clinician, the isolation of a new and highly active compound, that can be administered intravenously with no obvious signs of toxicity, is of some significance. These data form a basis for the continuing investigation of the biological activity of hyperimmune milk and of the claim of therapeutic efficacy in arthritis and other disease with an inflammatory basis.

Received 28 February 1990; accepted by F. Ahnfeld-Rønne 4 July 1990

References

[1] A. S. Goldman and C. W. Smith, *Host resistance factors in human milk.* J. Pediat. *82,* 1082–1090 (1973).

[2] R. C. Williams and R. J. Gibbons, *Inhibition of bacterial adherence by secretory immunoglobulin A: A mechanism of antigen disposal.* Science *177,* 697–699 (1972).

[3] C. Svanborg Edèn, B. Carlsson, L. A. Hanson, B. Jann, K. Jann, T. Korhonen and T. Wadström, *Anti-pili antibodies in breast milk.* Lancet *2,* 1235 (1979).

[4] A. S. Goldman, A. J. Ham Pong and R. M. Goldblum, *Host Defences: Development and maternal contributions.* Advances Pediat. *32,* 71–100 (1985).

[5] A. S. Cunningham, *Breast-feeding and health.* J. Pediat. *110,* 658–659 (1988).

[6] W. E. Owens and C. S. Nickerson, *Evaluation of an anti-inflammatory factor derived from hyperimmunized cows.* Proc. Soc. Exp. Biol. Med. *190,* 79–86 (1988).

[7] B. Campbell and W. E. Petersen, *Immune milk – a historical survey.* Dairy Science Abstracts *25,* 345–358 (1963).

[8] C. A. Winter, E. A. Risley and G. W. Nuss, *Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs.* Proc. Soc. Exp. Biol. Med. *111,* 544–547 (1962).

[9] R. J. Stolle and L. R. Beck, *Prevention and treatment of rheumatoid arthritis.* United States patent number 4,732,757 (1988).

[10] L. R. Beck, *Method of treating inflammation using bovine milk.* United States patent number 4,284,623 (continuation in part, in press, 1990).

[11] B. Campbell and W. E. Petersen, *Immune milk: The current picture.* J. Immune Milk *1,* 3–28 (1964).

[12] B. Campbell and W. E. Petersen, *Use of protective principles in milk and colostrum in prevention of disease in man and animals.* Lancet *75,* 494–501 (1955).

[13] C. M. Smith, *Rheumatoid arthritis syndrome: A statistical study based on replies to a questionnaire.* J. Immune Milk *1,* 37–42 (1964).

[14] A. S. Goldman, L. W. Thorpe, R. M. Goldblum and L. A. Hanson, *Anti-inflammatory properties of human milk.* Acta. Paediatr. Scand. *75,* 689–695 (1986).

[15] W. C. Lo, *Human milk: Nutritional properties.* In *Nutrition in paediatrics* (Eds W. A. Walker and J. B. Watkins) pp. 797–818, Little, Brown and Company, Boston 1985.

[16] M. E. Roux, M. McWilliams, J. M. Phillips-Quagliata, P. Weisz-Carrington, M. E. Lamm, *Origin of IGA-secreting plasma cells in the mammary gland.* J. Exp. Med. *146,* 1311–1322 (1977).

[17] R. M. Goldblum, S. Ahlstedt, B. Carlsson, L. A. Hanson, U. Jodal, G. Lidin-Janson and A. Sohl-Akerlund, *Antibody forming cells in human colostrum after oral immunization.* Nature *257,* 797–798 (1975).

[18] M. Fishaut, D. Murphy, M. Neifert, K. McIntosh and P. L. Ogra, *Bronchomammary axis in the immune response to respiratory syncytial virus.* J. Paediatr. *99,* 186–191 (1981).

[19] R. A. Allardyce, D. J. C. Shearman, D. B. L. McClelland, K. Marwick, A. J. Simpson and R. B. Laidlaw, *Appearance of specific colostrum antibodies after clinical infection with Salmonella typhimurium.* B. M. J. *3,* 307–309 (1974).

[20] B. A. Peri, C. M. Theodore, G. A. Losonsky, J. M. Fishaut, R. M. Rothberg and P. L. Ogra, *Antibody content of rabbit milk and serum following inhalation or ingestion of respiratory syncytial virus and bovine serum albumin.* Clin. Exp. Immunol. *48,* 91–101 (1982).

[21] P. C. Montgomery, K. M. Connelly, C. Cohen and C. A. Skandera, *Remote-site stimulation of secretory IgA antibodies following bronchial and gastric stimulation.* Adv. Exp. Med. Biol. *107,* 113–122 (1978).

[22] P. C. Montgomery, C. Cohen, C. A. Akandera and K. M. Connelly, *Evidence for an IgA anamnestic response in rabbit mammary secretions.* In *Immunology of breast milk.* (Eds. P. L. Ogra and D. H. Dayton) pp. 115–130, Raven Press, New York 1979.

[23] R. J. Love and B. M. Ogilvie, *Nippostrongylus brasiliensis and Trichinella spiralis: Localization of lymphoblasts in the small intestine of parasitized rats.* Exp. Parasitol. *41,* 124–132 (1977).

[24] M. A. Keller, R. M. Kidd, Y. J. Bryson, J. L. Turner and J. Carter, *Lymphokine production by human milk lymphocytes.* Infect. Immun. *32,* 632–636 (1981).

[25] G. Emodi and M. Just, *Interferon production by lymphocytes in human milk.* Scand. J. Immunol. *3,* 157–160 (1974).

[26] P. C. Canning and J. D. Neil, *Isolation and characterization of interleukin-1 from bovine polymorphonuclear leucocytes.* J. Leucocyte Biol. *45,* 21–28 (1989).

[27] J. W. M. Lawton, K. F. Shortridge, R. L. C. Wong and M. H. Ng, *Interferon synthesis by human colostral leucocytes.* Arch. Dis. Childhood. *54,* 127–130 (1979).

[28] H. Blau, J. H. Passwell, M. Levanon, J. Davidson, F. Kohen and B. Ramot, *Studies on human milk macrophages: Effect of activation on phagocytosis and secretion of prostaglandin E_2 and lysozyme.* Paediatr. Res. *17,* 241–245 (1983).

[29] B. Reid, H. Smith and Z. Friedman, *Prostaglandins in human milk.* Paediatrics. *66,* 870–872 (1980).

[30] F. S. Cole, E. E. Schneeberger, N. A. Lichtenberg and H. R. Colten, *Complement biosynthesis in human breast-milk macrophages and blood monocytes.* Immunology *46,* 429–440 (1982).

[31] C. H. W. Horne, S. S. Armstrong, A. W. Thomson and W. D. Thompson, *Detection of pregnancy associated α_2-glycoprotein (α_2-PAG), an immunosuppressive agent, in IgA producing plasma cells and in body secretions.* Clin. Exp. Immunol. *51,* 631–638 (1983).

[32] A. B. Sabin and A. H. Fieldsteel, *Antipoliomyelitic activity of human and bovine colostrum and milk.* Paediatrics. *29,* 105–115 (1962).

[33] R. H. Michaels, *Studies of anti-viral factors in human milk and serum.* J. Immunol. *94,* 262–271 (1965).