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(54) A PROCESS FOR THE PRODUCTION OF A PROTEIN CONCENTRATE CONTAINING IMMUNOLOGICAL FACTORS OF LACTIC ORIGIN

(71) We, SOCIETE DES PRODUITS NESTLE S.A. a Swiss body corporate of Vevey, Switzerland, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:

This invention relates to a process for the production of a protein concentrate containing immunological factors of lactic origin, particularly active immunoglobulins.

It has already been proposed to introduce immunological factors of lactic origin into dietetic products for new born babies and infants by incorporating these factors in their milk, the oral injection of these products being intended to enable these factors to be transferred to the blood of the infant, but without any indication of the methods adopted for or the results of such generalised passive immunisation.

It has also been proposed to isolate immune lactoglobulins from the milk of vaccinated cows by coagulating the milk, recovering the lactoserum and selectively precipitating the lactoglobulins with ammonium sulphate, followed by dialysis against pure water, filtration and drying. Seroprotection tests in mice in conjunction with these works have shown that the parenteral injection of these immunising principles afforded generalised passive protection against certain enteropathogenic bacteria and viruses when the animals were injected with these microorganisms by the same route.

The present applicants have now found a process for producing a protein concentrate containing immunoglobulins on a commercial scale without the immunoglobulins being denatured during the technological process. This product provides local passive immunity at the level of the intestine without resorption and without any significant loss of activity in the digestive tract.

The process according to the invention comprises the following steps:

- collecting the milk of hyperimmunised milk-bearing females,
- separating the cream and the impurities, coagulating the clarified and skimmed milk, separating the casein, filtering, ultrafiltering and sterilising the proteins of the whey by filtration, evaporating and drying the product under conditions which do not denature the immunoglobulins and which maintain sterility.

The accompanying drawing is a diagrammatic illustration of the various steps of the process.

The milk-bearing females used are bovidae, particularly cows.

The milk to be treated may be of various types more or less rich in antibodies: colostrum, transition milk (milk from the 8 days after calving) or end-of-lactation milk (last 2 months of lactation).

It is preferred to treat a mixture of these milks to obtain a high antibody level for the longest possible period.

The cows are hyperimmunised by combined vaccination, for example by intracisternal instillation into the mammary gland, parenteral injection (subcutaneous, intravenous), injection into the retromammary ganglionic system, by scarification, by oral ingestion or by a combination of several of these modes.

It is preferred to use a combination of these modes in an immunisation plan carefully drawn up to obtain a satisfactory antibody level in each type of milk used. Thus, in the case of colostrum and transition milk, vaccination is by the parenteral and intracisternal route in the 8-2 weeks before calving and by the oral route during the week preceding calving.

8

1,573,995

2

In the case of end-of-lactation milk, parenteral, local and oral vaccination are applied in alternation over a period of 2 and a half months before the presumed end of lactation.

The vaccine used is advantageously a mixture of the selected antigen and an adjuvant based on the homologous blood serum of immunised cows, thus providing for detoxication of the vaccine and for the formation of immune complexes.

Since cows are used as source animals, the Ig's contained in the protein concentrate are primarily G₁Ig's (particularly G₁Ig's), except for maternal milk in which A Ig's are the preponderant Ig's.

The Ig-content in the protein concentrate, in which the proteins represent from 70 to 80% by weight, amounts to between 25 and 35% by weight. Certain advantages are associated with the fact that, in the practical application of the process, the Ig's are not separated from the other proteins of the lactosera which, therefore, are also present in the concentrate obtained. Accordingly, there is no longer any need for the operations of selected precipitation, for example with ammonium sulphate, and dialysis.

In addition, certain bacteriostatic or antiviral factors, for example lactoferrin, or the enzymatic systems, such as lactoperoxidase and ribonuclease, which are present in the lactosera are also found in the protein concentrate.

All kinds of antigens of viral or bacterial origin may be used and the antibodies obtained are dependent upon the nature of the antigens administered to the cow. For example, it is possible to obtain protection against the enteropathogenic bacteria and viruses which are responsible for gastroenteritis, accompanied by serious diarrhoea and dehydration, by incorporating the protein concentrate containing the Ig's in any support suitable for oral administration. This support may be formed by any excipient or, preferably, by a dietetic product such as a milk or a malted milk for babies, a milk which has been "humanised", i.e. adapted for infants, a milk which has been specially adapted for a particular group of new born babies, such as for example a milk for premature babies or babies with a low birth weight, etc.

In cases where it is desired for example to use a concentrate containing anti-E.coli Ig's in a prophylactic dose with a milk as support, from 0.8 to 3 g of concentrate will be incorporated in 100 g of dry material, a quantity of up to 6 g of concentrate being sufficient for a therapeutic dose.

For carrying out the process, it is necessary to insure that the skimming and clarifying operations are extremely thorough to avoid subsequent blockage of the filters and ultrafilters.

Skimming is preferably carried out in two stages, firstly (at a lower temperature) for eliminating most of the fats and impurities (such as the blood often present in colostrum) and then (at a higher temperature) for completely separating the fats and impurities. Coagulation may be carried out at the isoelectric pH of casein in the presence of rennet with addition of an acid, for example citric acid.

It is preferred to carry out coagulation by the addition of an acid, for example hydrochloric acid, up to a pH-value of approximately 4.6 and by heating to a temperature of 40°C.

Separation of the casein is accompanied by clarification of the serum enabling the fines to be separated.

On the other hand, it is important to carry out the operations requiring heat treatment (evaporation and drying) under conditions designed to prevent inactivation of the Ig's.

In order to reduce the losses of Ig's, some of which are eliminated with the cream during the skimming operation and others with the casein during the separation thereof, it is advisable to extract the cream and the curd with water and to treat the washing waters containing the Ig's in order to recover them.

The process according to the invention is illustrated by the following Examples in which the quantities and proportions quoted are by weight, unless otherwise indicated.

EXAMPLE 1

Cows of various species were hyperimmunised in accordance with the following plan using a vaccine of which the preparation is described hereinafter, and the milk from the last two months of lactation and from the first 8 days after calving was collected.

Preparation of the vaccine

The following enteropathogenic E.coli serotypes were used:

0 18 : K 76 (B 20)

0 20 : K 17 (L)

0 26 : K 60 (B 6)

0 44 : K 74 (L)

0 55 : K 59 (B 5)

0 78 : K 80 (-)

0 86 : K 61 (B 7)

0 111 : K 58 (B 4)

0 112 : K 68 (B 11)

0 119 : K 69 (B 14)

0 124 : K 72 (B 17)

0 125 : K 70 (B 15)

0 126 : K 71 (B 16)

0 127 : K 63 (B 8)

0 128 : K 68 (B 12)

(9)

The various strains originated from cases of gastroenteritis in hospitalised infants. Serotyping was carried out with multivalent and monovalent anti-sera (Difco the work "Difco" being a Registered Trade Mark). The bacteria were cultivated in minimal liquid medium containing 2% of amino acids derived from casein (Difco casamino acids) and 2% of glucose for 24 hours at +37°C without agitation of the bottles. In order to inactivate the germs, the cultures were separated by centrifuging into sedimented cells and supernatant phase. The cells were resuspended and incubated for 24 hours at +37°C in the presence of 0.5% of formaldehyde, whilst the supernatant phase was inactivated as described above, but with 0.05% of formaldehyde. After recentrifuging and elimination of the supernatant phase, the bacteria were resuspended in the original inactivated supernatant phase. This procedure enables the supernatant phase containing bacterial exotoxins to be conserved for the vaccine. A vaccine containing $1-2 \times 10^9$ bacteria/ml was obtained and was subsequently diluted by mixing with a 2% solution of $Al(OH)_3$ and homologous blood serum of immunised cows.

Immunisation procedure

A. Immunisation plan for obtaining colostral and transition milk containing anti-E.coli Ig's:

Presumed calving date = X

Time of treatment	Vaccine + adjuvant (if any)	Method of administration
X - 8 weeks	5 ml of vaccine (5×10^7 bacteria/ml) + 5 ml of serum *	subcutaneous injection
X - 7 weeks	5 ml of vaccine (5×10^7 bacteria/ml) + 5 ml of 2 % $Al(OH)_3$ + 5 ml of serum *	intravenous injection
X - 6 weeks	10 ml of vaccine (5×10^7 bacteria/ml)	subcutaneous injection
X - 5½ weeks	20 ml of vaccine (5×10^7 bacteria/ml) + 10 ml of 2 % $Al(OH)_3$	injection into the retromammary ganglionic system
X - 5 weeks	40 ml of vaccine (5×10^7 bacteria/ml) + 20 ml of serum	instillation of 4 x 15 ml into the udder through the teat passages
X - 4½ weeks	24 ml of vaccine (5×10^8 bacteria/ml) + 8 ml of serum *	subcutaneous injection **
X - 4 weeks	40 ml of vaccine (5×10^7 bacteria/ml) + 20 ml of serum	instillation of 4 x 15 ml into the udder through the teat passages
X - 3 weeks	10 ml of vaccine (5×10^8 bacteria/ml)	subcutaneous injection
X - 2 weeks	5 ml of vaccine (5×10^8 bacteria/ml) + 5 ml of 2 % $Al(OH)_3$	intravenous injection
X - 1 week	100 ml of vaccine (1×10^9 bacteria/ml)	oral ingestion during rumination
X - ½ week	100 ml of vaccine (1×10^9 bacteria/ml)	oral ingestion during rumination

(10)

48

1,573,995

48

* These additions of serum are only made where a cow is being immunised for the first time.

** This subcutaneous injection is distributed as follows throughout the lymphatic system:

- 5: 2 x 4 ml in the pre-scapular lymphatic ganglions
2 x 4 ml in the post-scapular lymphatic ganglions
2 x 4 ml in the inguinal lymphatic ganglions
2 x 4 ml in the popliteal lymphatic ganglions

B Immunisation plan for obtaining end-of-lactation milk (last 2 months of lactation) containing anti-E.coli Ig's.

- 10: This immunisation applies solely to cows which have already been immunised for the colostral and transition periods.

Presumed end-of-lactation date = X

Time of treatment	Vaccine + adjuvant (if any)	Method of administration
X-70 days	5 ml of vaccine (5×10^7 bacteria/ml) + 5 ml of 2 % Al (OH) ₃	intravenous injection
X-65 days	20 ml of vaccine (5×10^8 bacteria/ml)	injection into the retromammary ganglionic system
X-60 days	24 ml of vaccine (5×10^8 bacteria/ml)	subcutaneous injection ** (as above in A)
X-55 days	100 ml of vaccine (1×10^9 bacteria/ml)	oral ingestion during rumination
X-50 days	40 ml of vaccine (5×10^7 bacteria/ml) + 20 ml of serum	instillation into the udder as above in A
X-40 days	100 ml of vaccine (1×10^9 bacteria/ml)	oral ingestion during rumination
X-30 days	20 ml of vaccine (5×10^8 bacteria/ml)	injection into the retromammary ganglionic system
X-25 days	40 ml of vaccine (5×10^7 bacteria/ml) + 20 ml of serum	instillation into the udder as above in A
X-10 days	100 ml of vaccine (1×10^9 bacteria/ml)	oral ingestion during rumination

(11)

EXAMPLE 2

Milk from the first 8 days after calving collected from cows vaccinated in accordance with Example 1 was treated as follows:

- skimming is carried out in two steps: step A at low temperature and step B at high temperature.

A) 1100 kg of cold milk enter a centrifugal skimmer and are directed towards a 2500 litre capacity buffer tank and are then pumped into a high-acceleration (approximately 11000 G) centrifugal clarifier so as to eliminate blood and impurities (dregs).

B) The cold, clarified milk is stored in a 2500 litre capacity reservoir and pumped through a heater tempered to 40°C into the centrifugal skimmer previously used, thus separating 110 kg of cream, which are recovered, and 5 kg of dregs which are destroyed. In a variant of this procedure, it is possible to use a self-clarifying skimmer for the low temperature and high temperature skimming operations and a clarifier between these operations and thus to reduce the skimming time.

- The hot clarified skimmed milk (985 g) is then distributed into 4 square 750 litre capacity coagulation tanks equipped with stirrers for the coagulating operation.

A 1 N hydrochloric acid solution is added at 37°C until the pH stabilises at around 4.6, after which the temperature is maintained for 20 minutes with stirring, followed by cooling to around 15°C.

- The following operation is separation of the casein. It consists of two centrifugings in series of the skimmed, curdled and slabbed milk. The first separates most of the curdled casein by means of a self-cleaning clarifier separating 150 kg of casein, the serum entering a buffer reservoir. The second centrifuging operation removes every trace of particles which could interfere with the following operation of filtration and ultrafiltration, for example by means of the clarifier used in step A of the skimming operation. 854.7 kg of clarified serum are obtained and are pumped into a 2500 litre capacity reservoir.

- The serum is then successively subjected to filtration and ultrafiltration. Filtration has to be very thorough to avoid difficulties during the ultrafiltration step by separating the very fine particles of casein. Filtration is carried out with a Seitz Supra 100 ("Seitz" is a Registered Trade Mark) filter comprising plates measuring 20 x 20 cm. The filtrate is stored in a 3000 litre capacity reservoir and is then pumped to the ultrafilter. Ultrafiltration is carried out in 2 or 3 steps with recycling of the retentate into the reservoir. Two centrifugal pumps arranged in series effect delivery against a pressure on entry into the input compartment of the ultrafilter of approximately 7 bars, at the output end of which the pressure is kept at around 4.5 bars. In order to prevent the retentate from being heated by the energy supplied to the pumps, it is cooled to approximately 10 - 12°C.

The first step, i.e. ultrafiltration proper, separates the solutes of high molecular weight and the proteins retained on the membrane, which are found in the retentate, whilst part of the lactose, the vitamins, mineral salts and water are removed in the permeate. By using a DDS module with an 800 membrane (membrane surface = 7m²), the retentate/permeate ratio being approximately 1 to 3.1, there are thus obtained 580 kg of permeate I and 274.7 kg of retentate with an average output of permeate of 14.17 kg/m²h.

The second step is a diafiltration, during which 825 kg of water are continuously added to the retentate and the solution is circulated under the same conditions as in the first step. Diafiltration is stopped when the electrical conductivity of the permeate reaches the required value, the ratio of retentate to added water being approximately 1 to 3. 825 kg of permeate II and 274.7 kg of retentate are thus separated with an average output of 11.77 kg of permeate m²h. The third step is a concentration step which is optionally carried out by recycling the retentate to the membrane up to a dry matter content of approximately 10% by the elimination of 82.5 kg of permeate III. 192.2 kg of preconcentrated solution are thus obtained. In a variant, this third step is left out and, instead, the process progresses directly to the sterile filtration stage.

The sterile filtration step is an important step in the process as a whole because the heat treatments are not applicable for sterilisation because they result in denaturing of the Ig's. Sterile filtration frees the retentate from the micro-organisms which may still be present therein (most of them having already been eliminated during separation of the cream, the casein and the dregs). To prevent the sterilising filters from becoming blocked, clarification by centrifuging and triple filtration of the retentate are necessary. After clarification, the retentate is stored in a reservoir and passed through a filtration line comprising a prefiltration stage on three filters (Seitz Supra 100, EK, EKS) arranged in series, followed by sterile filtration on a Seitz Supra 20 filter and a Millipore HA (0.45 µ) filter ("Millipore" is a Registered Trade Mark) arranged in series and sterilised beforehand with superheated water. The sterile retentate is stored in a 500 litre capacity sterile reservoir.

All the following operations are carried out under sterile conditions. The transition to the following evaporation step may be made for example under sterile, compressed and filtered

nitrogen.

The evaporation step is carried out in a thin layer evaporator (with a falling film having a surface area of 1 square metre) at a heating temperature of 75°C, the temperature of the product being approximately 30°C, up to a dry matter content of at least 20%. In order to avoid infection, the transfer is made by peristaltic pump from the retentate reservoir to the concentrate reservoir which is connected to a loop comprising the evaporator, the concentrating operation taking place in a closed circuit. This operation does not affect the activity of the Ig's.

The concentrate (96 g) is then dried by any suitable means, for example by freezing followed by freeze-drying. Freezing may be carried out on plates at -40°C. The Ig's may be stored at -30°C for about 45 days without any loss of activity. The product at -30°C is freeze-dried on plates in a chamber under 0.5 Torr, with a temperature of the condenser of -50°C, and heated to a temperature of from 30 to 35°C. The method used for drying does not affect the activity of the Ig's. 19.1 kg of dry product containing from 25 to 35% of Ig's are thus obtained, being packaged under sterile conditions.

EXAMPLE 3

By adopting the procedure described in Example 2 for treating milk from the first 8 days after calving and milk collected during the last four weeks of lactation, a protein concentrate having the following average composition is obtained:

Proteins	75 ± 5%
30 ± 5% of immunoglobulins	
15 ± 5% of α-lactalbumins	
35 ± 5% of β-lactoglobulins	
5 ± 2% of serum albumins	
5 ± 2% of other minor proteins	
residual moisture	4 ± 0.5%
lactose	10 ± 2%
mineral salts	5 ± 2%
non-protein nitrogen-containing substances	5 ± 2%

EXAMPLE 4

The procedure is as in Example 2, except that the cream and the casein are extracted with water to recover some of the lost Ig's, the washing waters being recycled into the production line.

Cold skimming (step A) is carried out in a parallel water extraction line for extracting the fraction of proteins containing the Ig's entrained with the cream. The cream emanating from the first centrifuging operation (115 kg) is stored in a 1000 litre capacity reservoir equipped with a stirrer and mixed with 150 kg of luke warm water at 40°C, the whole being stirred for 30 minutes and centrifuged in a skimmer. 115 kg of cream and 150 kg of solution are separated in this way, the solution being recombined with the skimmed and clarified milk. 1130 kg of liquid are thus obtained for hot skimming (step B) and coagulation.

Separation of the casein gives 991 kg of serum and 154 kg of casein from 1145 kg of skimmed, renneted and acidified milk. A parallel line is used for extracting the fraction of proteins containing the Ig's from the curds with water. On leaving the self-cleaning clarifier, the curds are stored in a reservoir equipped with a stirrer and are stirred with 300 kg of luke warm water at 40°C for 30 minutes and delivered to a colloid mill. The suspension of ground curds is then separated in a self-cleaning clarifier. 154 kg of washed casein and 300 kg of washing liquid are recovered, the washing liquid being recombined with the serum for the subsequent operations. 1291 kg of serum are filtered and ultrafiltered, giving 2010 kg of permeates (I, II and III) and 216 kg of pre-concentrate. Evaporation gives 108 kg of concentrate which in turn gives 21.6 kg of dry product by drying. This represents an increase of approximately 14% of Ig's over the process described in Example 2.

EXAMPLE 5

1 kg of the powder prepared in accordance with Example 2 or 4 or 2 kg of the powder prepared in accordance with Example 3 is homogeneously mixed with 100 kg of milk powder which is then packaged under sterile conditions to give a product containing a prophylactic dose of Ig's for an isocaloric feed of 140 cal/kg/day corresponding to 250 mg of Ig-concentrate/kg/day prepared in accordance with Example 2 or 4 or to 500 mg of Ig-concentrate/kg/day prepared in accordance with Example 3.

EXAMPLE 6

Various *in vitro* and *in vivo* tests were carried out with the protein concentrate according to Example 2 or 4, hereinafter referred to as "Ig-concentrate", to demonstrate

- that the lactic Ig's have an antibody specificity which is normally found in human Ig's and that this specificity is maintained during the production process,
- that the specific lactic Ig's show protective activity by interfering in the pathogenic mechanism of enteropathogenic microorganisms.

Passive haemagglutination

Erythrocytes from sheep were sensitised with a urea extract of a specific E.coli serotype. This extract was obtained by Brodhage's method (Brodhage H., (1961) J. Hyg., 1961, 148, 94). The bacteria were cultivated for 30 hours at 37°C on nutrient agar (Difco) in Roux bottles. The culture was harvested with 10 ml of saline solution buffered with phosphate (8.8 g NaCl + 1.86 g Na₂HPO₄·2H₂O + 0.43 g KH₂PO₄/1000 ml H₂O) to pH 7.2. Following the addition of 10 g of urea (Merk), the suspension was slowly stirred for 90 hours at 37°C. After centrifuging, the supernatant phase was completely dialysed against a saline solution buffered with phosphate to pH 7.2, diluted to a final volume of 50 ml with saline solution buffered with phosphate to pH 7.2 and frozen in small portions at -20°C. The sheep's erythrocytes were sensitised with this antigen by a combination of the methods of Avrameas et al (Avrameas S., Taudou B., Chuilon S., Immunochemistry, 1969, 6, 67) and Otto et al (Otto H., Takamiya H., Vogt A., J. Immunol. Methods. 1973, 3, 137). The sheep's erythrocytes were pretreated with glutaraldehyde (final concentration of sheep's erythrocytes 5% and glutaraldehyde 0.5 %), washed, suspended in saline solution buffered with phosphate to pH 7.2 to form a 20% suspension and then mixed with the same volume of urea extract. After incubation for 16 hours at 20°C with gentle stirring, the sensitised sheep's erythrocytes were washed several times and suspended to a level of 1% for immediate use. In the form of a 10% suspension, the sheep's erythrocytes may be stored at 4°C for several weeks.

Before filtration, each samples to be examined has to be absorbed onto sheep's erythrocyte pretreated with glutaraldehyde (0.1ml of sedimented sheep's erythrocyte for 1 ml of Ig-concentrate, 37°C, 60 minutes).

The titrations were carried out under the Microtiter system (Sever J.L., J. Immunol., 1962, 88, 320 and Conrath T.B., Handbook of Microtiter Procedures, 1972) using polystyrene plates formed with V-shaped cavities. A series of 50 µl dilutions in normal rabbit's serum diluted to 1/200 were prepared and 25 µl of 1% sensitised sheep's erythrocyte were added to each dilution. Another series was prepared with non-sensitised sheep's erythrocytes as non-specific agglutination control. The results were read off after 2 hours, the haemagglutination titer being given by the last dilution leading to a clear positive reaction.

The results obtained for 5 E.coli serotypes are given in the following Table:

E.coli serotype	agglutinin titer
0 55	1/256
0 111	1/64
0 119	1/128
0 127	1/128
0 128	1/64
control	—

(124)

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Bacteriostatic activity in vitro

The bacteriostatic activity on the growth of various *E. coli* serotypes was studied by adding Ig-concentrate to the culture medium. The microtiter system adapted for the culture was used to enable minimal quantities of sample to be used for testing and a maximum number of samples to be examined at the same time.

Each culture had a final volume of 0.25 ml either in a minimal medium containing 1% of glucose or in a rich culture medium. For each value of the incubation time studied, two samples were taken in order to reduce the risk of error attributable to variations in volume. The results were evaluated by the double counting of 10 μ l aliquots on plates of nutrient agar. The bacterial inoculum consisted of a dilution of a 16 hour culture in minimal medium containing 1 to 2 x 10³ bacteria/ml. Incubation was carried out in a humid atmosphere at a temperature of 37°C. The Ig-concentrate in the final concentrations of 1 μ g/ml, 10 μ g/ml, 100 μ g/ml and 1 mg/ml was added to the culture medium before inoculation with 2 x 10³ *E. coli*/ml. With the 10 μ g/ml concentration of Ig-concentrate, a distinct inhibition of the growth of *E. coli* 0 111 : B4 was observed during the first 4 hours of incubation. By comparison with the control formed by the culture medium on its own, greater growth was observed in the presence of 1 mg/ml of normal whey proteins obtained from non-immunised cows.

Clearance of E. coli 0 111: B4 in mice

8 Mice (male Swiss white mice weighing from 20 to 24 g) were used for each test, 2 as controls and 6 for 3 tests each carried out twice. All the mice were subcutaneously injected with 0.1 ml of heparin (500 UI/ml). A 16 hour culture broth of *E. coli* 0 111: B4 was diluted in a ratio of 1:10 with a sterile saline solution and then in a ratio of 1:100 by the addition of foetal calf serum (Difco) diluted in a ratio of 1:10 for the controls and containing three selected concentrations of Ig-concentrate. 0.2 ml of the solution obtained was intravenously injected into the caudal vein, each mouse (2 mice for the control and 2 x 3 for the tests) receiving approximately 2 x 10⁶ bacteria, the original bacterial suspension containing 10⁹ bacteria/ml. Immediately after the injection (at time t₀) and after 20, 40 and 60 minutes, 50 μ l of blood was removed from the ophthalmic venous plexus by the insertion of calibrated heparin-lined capillary tubes, and the samples were immediately transferred to 5 ml of sterile saline solution (blood dilution 10⁻²) which was then diluted to 10⁻³ and 10⁻⁴ in saline solution. Counting was carried out on agar plates (Difco) with 1 ml of each dilution and the phagocytosis index K was determined in accordance with Biozzi et al (Biozzi G., Stiffel G., Halpern B.M., Le Minor L., Mauton D., J. Immunol., 1961, 87, 296):

$$K = \frac{\log C_1 - \log C_2}{T_2 - T_1}$$

C₁ and C₂ correspond to the counts at times T₁ and T₂.

In this way, there was observed a normal reduction in the number of bacteria by elimination in the reticulo-endothelial system in the presence of foetal calf serum, whereas the addition of 10 μ g of Ig-concentrate to the injection solution resulted in the disappearance of 99.6% of the bacteria from the bloodstream over a period of 20 minutes.

The results are set out in the following Table:

Time (minutes after the injection of <i>E. coli</i>)	bacteria in % of the initial number at time 0 after the intravenous injection of 2x10 ⁶ <i>E. coli</i> 0 111: B4			
	Control 1:10 foetal calf serum	Tests, Ig-concentrate		
		1000 μ g	100 μ g	10 μ g
0	100	100	100	100
20	27.8	0.14	0.22	0.4
40	15.5	0.02	0.02	0.12
60	10.0	0.02	0.02	0.06

Tests were conducted to determine the specificity of the opsonisation process by the exhaustive absorption of Ig-concentrate with the 0 111:B4 serotype of E.coli and showed that this absorption destroys practically all the activity in increasing phagocytosis, even with a quantity of 1 mg of Ig-concentrate.

5 The following Table shows the phagocytosis indices K obtained after 20 minutes: 5

	Control 1:10 foetal calf serum	1000 g of non- absorbed Ig- concentrate	1000 g of absorbed Ig-concentrate
K	0.015	0.128	0.039

Protection test in mice

Logarithmic dilutions ($\log_{10}$) of a culture of E.coli 0 111:B4 in nutrient broth (Difco) containing 10^9 bacteria/ml were prepared to give 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} dilutions in 5% mucin (granular mucin, type 1701-W for potentiating the virulence of bacteria, a product of Wilson Laboratories, Chicago, Illinois).

3 ml of each dilution were then mixed with 0.6 ml of solution to be tested, i.e. respectively saline solution, Ig-concentrate and normal whey proteins (non-immunised cows). Using 5 mice for each dilution, 0.6 ml of the mixture obtained was intraperitoneally injected into each mouse. The results of the evaluation of the number of dead mice out of 5 mice treated after 48 hours are set out in the following Table.

Material tested	Number of dead mice/5 mice treated			
	Bacteria concentration of the treatment			
	5×10^4	5×10^3	5×10^2	5×10^1
Saline solution	5	5	5	5
10 μ of Ig-concentrate *	5	5	5	3
25 μ of Ig-concentrate *	5	4	0	0
100 μ of Ig-concentrate *	0	0	0	0
10 μ of normal whey proteins (non- immunised cows)	5	5	5	5
25 μ of normal whey proteins	5	5	5	5
100 μ of normal whey proteins	5	5	5	5

* calculated as total proteins containing from 35 to 45% of lactic Ig's.

It was found that 100 μ g of Ig-concentrate afforded complete protection for all the bacteria concentrations tested, whilst 100 μ g of whey proteins isolated from the milk of non-immunised cows afforded no protection.

EXAMPLE 7

1) A first series of clinical tests show that the lactic Ig's withstand proteolytic degradation into inactive fragments in the intestinal tract.

11 infants (9 boys, 2 girls) ranging in age from 2 weeks to 1 year, hospitalised for various reasons, but not suffering from gastroenteritis, were fed on a milk containing an Ig-concentrate prepared in accordance with Example 2 or 4 in an isocaloric dose corresponding to 2 g/kg of body weight equally distributed over 24 hours. The first and last feeds incorporating Ig-concentrate were marked by 0.2 g of carmine red. At least one stool was collected before the administration of Ig-concentrate and all the following stools were systematically collected over a period of 72 hours, the stools being stored at -20°C .

Extracts of the stools were prepared by the addition of 2 parts of stool to 1 part of saline

The presence of active Ig's in the stool extracts was confirmed by double immunodiffusion (Ouchterlony's test) and by immunoelectrophoresis. The antisera used in these tests were prepared by the repeated subcutaneous and intravenous injection in rabbits of 1 mg of lactic antibody or 0.1 mg of G₁Ig. For the Ouchterlony test, glass plates (94 x 84 mm) were coated with a layer of 1% of agar gel in a saline solution buffered with phosphate to pH 7.2, in which 4mm cavities separated by intervals of 9.5 mm had been formed by means of a punch (LKB-Producteur AB), using an antiprotein antiserum of bovine colostral whey and a monovalent specific anti-G₁Ig antiserum of cows milk.

20 Lactic Ig's were found to be present in the stools despite considerable variations in the moment of their appearance and in the duration of the Ig-secretion. There was also good correspondence between the appearance and disappearance of the carmine red marker and the Ig's.

25 being set out in the following Table:

Infant M.D. (2 weeks)	I	—	5	5	5	5
	II	—	5	5	5	5
	IV	+ + + +	2	1	0	0
	V	+ + + +	0	0	0	0
	VI	+ + +	1	1	0	0
	IX	—	5	5	3	4
Infant S.G. (1 month)	I	—	5	5	5	5
	III	—	5	5	5	5
	IV	+ + + +	0	0	0	0
	VI	+ + + +	0	0	0	0
	X	+ +	3	0	1	0
	XI	+ +	5	2	0	0

30 II) A second series of clinical tests was carried out to determine the therapeutic and prophylactic properties of the Ig-concentrate prepared in accordance with Example 2 or 4.

35 through their food either 2 g of Ig-concentrate/kg/day for 5 days or 1 g/kg/day for 10 days. The patients were not given any medicaments, such as sulphamides or antibiotics, either orally or parenterally. Coprocultures were prepared the day after each administration, the last between 36 and 48 hours after the final administration of Ig-concentrate. 43 patients were treated in the same way but with whey proteins from non-immunised cows instead of the Ig-concentrate.

40 Ig-concentrate. Negative coprocultures were obtained for 90 infants (57.7%) during the treatment. In the

control series, only 14 infants (27.7%) showed a spontaneous disappearance of E.coli in the coprocultures. Allowance must be made for the fact that the native proteins of whey from non-immunised cows contain a small quantity of anti E.coli Ig. It was also found that the treatment was unsuccessful more frequently in those cases where the highest dose had been administered over a shorter period.

The consistency of the stools was examined in each case. Examination showed the disappearance of diarrhoea and a significant clinical improvement in a large number of cases, even when the coprocultures remain positive. In cases where the coprocultures were negative, the consistency of the stools improved more rapidly when the Ig-concentrate was administered in a milk formulation of low lactose content.

Prophylactic properties

Premature babies are an extremely sensitive group so far as gastroenteritis by E.coli is concerned. It was this group that was selected for the clinical prophylaxis tests.

Each study was carried out over a period of 6 months in 2 rooms with 8 incubators in each room. The tests and the controls in each room were distributed in such a way that all the patients were exposed to the same conditions, 4 incubators being used for the test and 4 for control purposes. Each patient stayed for an average of 41 days, an infant being removed after recovery and replaced by another, irrespective of its group (test or control). 70 cases were followed, 36 as controls and 34 for the test group.

The test group received the Ig-concentrate at each feed distributed over 24 hours in a quantity of 0.25 g/kg/day. Coprocultures were prepared from the beginning of the test and examined every 5 days. Of a total of 666 coprocultures, 339 belonged to the control group and 327 to the test group. Of the latter, 33 showed the presence of E.coli (10.1%), whilst 96 of the 339 control cultures were positive (28.3%). When the serotype E.coli 0 119:B14, which is often associated with acute forms of gastroenteritis, was examined on its own, the frequency of positive coprocultures was 5.5% in the test group as opposed to 23.3% in the control group.

It may be concluded that the incorporation in milk for premature babies of protein concentrate containing specific anti E.coli lactic Ig's distinctly reduces the degree of infection by E.coli in this particularly sensitive group of new born infants.

WHAT WE CLAIM IS:-

1. A process for the production of a protein concentrate containing immunological factors of lactic origin, particularly active non-denatured immunoglobulins, comprising the following steps:

collecting the milk of hyperimmunised milk-bearing females
separating the cream and the impurities.
coagulating the clarified and skimmed milk,
separating the casein, filtering, ultrafiltering and sterilising the proteins of the whey by filtration, evaporating and drying the product under conditions which do not denature the immunoglobulins and which maintain sterility.

2. A process as claimed in Claim 1, wherein the milk treated is milk from cows hyperimmunised by vaccination and consists of

- colostrum from the first and second day after calving,
- transition milk from the third to eighth days after calving,
- and end-of-lactation milk from the last 60 days of lactation.

3. A process as claimed in Claim 2, wherein the vaccination plan for colostrum and transition milk comprises a series of successive administrations of antigens by the parenteral and local route from about the eighth week to the second week before calving and by the oral route for about the last two weeks before calving and, for end-of-lactation milk, a series of successive administrations of antigen commencing approximately 2 and half months before the presumed end of lactation with alternation of the parenteral, local and oral routes.

4. A process as claimed in Claim 3, wherein the vaccine is based on antigens of *Escherichia coli* responsible for neonatal gastroenteritis.

5. A process as claimed in any of claims 1 to 4, wherein coagulation is carried out by adding hydrochloric acid up to a pH-value of approximately 4.6 and heating 40°C.

6. A process as claimed in any of claims 1 to 5, wherein to prevent subsequent blockage of the filters and ultrafilters, the skimming operation is carried out in two steps, the second step taking place at a higher temperature than the first, and wherein skimming and separation of the casein are accompanied by thorough clarification.

7. A process as claimed in any of claims 1 to 6, wherein ultrafiltration of the whey is carried out in three steps, namely ultrafiltration proper, diafiltration and preconcentration.

8. A process as claimed in claim 7, wherein the preconcentrated whey is subjected to prefiltration followed by sterile filtration.

9. A process as claimed in claim 7, wherein evaporation of the preconcentrated and sterilised whey takes place in a closed circuit under sterile conditions.



17

control series, only 14 infants (27.7%) showed a spontaneous disappearance of E.coli in the coprocultures. Allowance must be made for the fact that the native proteins of whey from non-immunised cows contain a small quantity of anti E.coli Ig. It was also found that the treatment was unsuccessful more frequently in those cases where the highest dose had been administered over a shorter period.

The consistency of the stools was examined in each case. Examination showed the disappearance of diarrhoea and a significant clinical improvement in a large number of cases, even when the coprocultures remain positive. In cases where the coprocultures were negative, the consistency of the stools improved more rapidly when the Ig-concentrate was administered in a milk formulation of low lactose content.

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It may be concluded that the incorporation in milk for premature babies of protein concentrate containing specific anti E.coli lactic Ig's distinctly reduces the degree of infection by E.coli in this particularly sensitive group of new born infants.

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1. A process for the production of a protein concentrate containing immunological factors of lactic origin, particularly active non-denatured immunoglobulins, comprising the following steps:

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2. A process as claimed in Claim 1, wherein the milk treated is milk from cows hyperimmunised by vaccination and consists of

- colostrum from the first and second day after calving,
- transition milk from the third to eighth days after calving,
- and end-of-lactation milk from the last 60 days of lactation.

3. A process as claimed in Claim 2, wherein the vaccination plan for colostrum and transition milk comprises a series of successive administrations of antigens by the parenteral and local route from about the eighth week to the second week before calving and by the oral route for about the last two weeks before calving and, for end-of-lactation milk, a series of successive administrations of antigen commencing approximately 2 and half months before the presumed end of lactation with alternation of the parenteral, local and oral routes.

4. A process as claimed in Claim 3, wherein the vaccine is based on antigens of *Escherichia coli* responsible for neonatal gastroenteritis.

5. A process as claimed in any of claims 1 to 4, wherein coagulation is carried out by adding hydrochloric acid up to a pH-value of approximately 4.6 and heating 40°C.

6. A process as claimed in any of claims 1 to 5, wherein to prevent subsequent blockage of the filters and ultrafilters, the skimming operation is carried out in two steps, the second step taking place at a higher temperature than the first, and wherein skimming and separation of the casein are accompanied by thorough clarification.

7. A process as claimed in any of claims 1 to 6, wherein ultrafiltration of the whey is carried out in three steps, namely ultrafiltration proper, diafiltration and preconcentration.

8. A process as claimed in claim 7, wherein the preconcentrated whey is subjected to prefiltration followed by sterile filtration.

9. A process as claimed in claim 7, wherein evaporation of the preconcentrated and sterilised whey takes place in a closed circuit under sterile conditions.

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10. A process as claimed in any of claims 1 to 9, wherein the concentrated product is freeze-dried and is packaged under sterile conditions.

5 11. A process as claimed in any of claims 1 to 10, wherein to recover the immunoglobulins eliminated during the operations of skimming and separation of the casein, the cream and the curds are extracted with water and the washing waters are respectively recombined with the skimmed milk and with the whey.

12. A process for the production of a protein concentrate substantially as described with particular reference to Examples 2 to 5.

10 13. A product rich in active non-denatured immunoglobulins when obtained by a process as claimed in any of claims 1 to 12.

14. A medicament against gastroenteritis caused by E.Coli in new born infants which comprises a product as claimed in claim 13 together with a pharmacologically acceptable carrier.

15 15. A medicament as claimed in claim 14 which also comprises a powdered or reconstituted milk.

16. A product when obtained by carrying out a process substantially as described with particular reference to any of Examples 2 to 5.

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