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PROCESS FOR THE MANUFACTURE OF PREPARATIONS RICH IN INTERFERON

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ABSTRACT OF THE DISCLOSURE

The invention provides a process for the manufacture of interferon-rich preparations by incubating human cells in the presence of an induction agent such as a virus, and separating the interferon formed.

This application is a continuation of copending application Ser. No. 635,356, filed May 2, 1967, now abandoned.

This invention relates to the manufacture of preparations rich in interferon. Infereron is a protein of very variable molecular weight synthesised by cells under the effect of external stimulations, the biological action of which consists in the intracellular blocking of viral multiplication, in an, as yet, undetermined manner. Interferon is specific for the cellular species in which it has been produced. For protecting human cells, in vitro it is therefore essential to employ an interferon prepared in human tissue. The interferons which are at present best known originate from preparations produced in tissues of chickens or mice. While their protective action against viral infections is indisputable, their curative power is less evident. The results appear to depend upon the quantity of interferon employed and upon the interval of time between the inoculation of the interferon and that of the infecting virus. The therapeutic value of interferon has not been sufficiently explored because considerable difficulties have hitherto been encountered in producing it in bulk.

This invention provides a convenient process for the manufacture of preparations rich in interferon which is specific for the human species, using starting materials which are available in substantially unlimited quantities.

The new process comprises incubating live human cells with an induction agent, separating the induction agent not fixed in the said cells, continuing the incubation of the infected cells, separating the interferon-containing supernatant liquid and adjusting the pH thereof to 6.9 to 7.6, preferably 7.2 to 7.4.

The cells used as starting material are preferably leucocytes, or cells derived from placental adnexa, notably amniotic membranes, chorions and placentas. These sources are substantially inexhaustible. It is also possible to start from other cells, e.g., human embryo cells.

Although any viral or nonviral induction agent may be used, it has been found that the best results are obtained with para-influenza virus, type I, Sendai strain.

When the starting cells are leucocytes, it has been found that it is advantageous to use cells derived from a variety of different donors, regardless of the blood groups to which they belong. More particularly, leucocytes may be isolated from a mixture of bloods protected from coagulation by a known technique, for example by the addition of sodium citrate. Thus, 75 ml. of a sterile, apyrogenic solution having the following composition:

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Citric acid	1.37
Sodium hydroxide	0.52
Glucose	2.50
Distilled water, to 75 ml.	

may be added to 400 ml. of blood.

The leucocytes may be separated by any known method, more particularly using agents which modify the speed of sedimentation. A particularly suitable mode of separation is as follows.

A mixture of citrated blood is added to a 6% solution of Dextran having a molecular weight of 180,000 in physiological saline, in a proportion of 4 volumes of blood to 1 volume of Dextran solution. After repeated agitation, this mixture is distributed in large separators made of silicone coated glass or a plastics material such as polytetrafluoroethylene. After standing for one hour, the sedimented red corpuscles are eliminated by the lower valve of the separator. The residual plasma, which contains all the leucocytes in suspension, is extracted through the top of the separator. All the plasma fractions from the various separators are combined after the leucocytes have been counted, and are further purified by centrifugation in an "International" centrifuge at 4° C., in oblique pots of plastics material, at 5,000 r.p.m. for 15 minutes. The supernatant liquids are decanted, kept and subsequently used in the preparation of interferon.

The strain of para-influenza virus type I, is preferably maintained by passing it through eggs of Warren and Leghorn breed hens, the inoculation taking place by the allantoidian route. In this manner, an allantoic liquid which contains, per tenth of a millilitre, from 10^8 to 10^9 times the 50% lethal dose, may be obtained.

The leucocytes which have been separated from the plasma by centrifuging still contain a residue of red corpuscles in a variable quantity. These corpuscles may be eliminated by causing their lysis by the virus used to infect the leucocytes and to initiate the production of interferon. More particularly, the leucocytes may be suspended in the virus-containing allantoic liquid at a pH of 7.2 to 7.3 (adjusted to this value by addition by hydrochloric acid or sodium hydroxide as needed), in a ratio such that there are 10 to 100 viral particles per leucocyte, and the mixture may then be incubated at 37° C. on the water bath with agitation. In a first phase, all the red corpuscles by which the preparation is contaminated agglutinate to form large clusters. In a succeeding phase, the clusters of red corpuscles gradually disappear by a process of lysis produced by the infecting virus. About 3 hours' incubation is required. The suspension of white corpuscles thus obtained may then be centrifuged and the less dense portion, containing the residual virus and the products of the lysis of the red blood corpuscles, may be eliminated.

The denser portion containing the infected leucocytes may then be incorporated in an Alsever solution containing about 10% of defibrinated human serum, until a homogeneous suspension is obtained. (The human serum is defibrinated, for example as follows: A 10% aqueous calcium chloride solution is added in a proportion of 30 ml. per litre to plasma collected at the time of the initial separation of the leucocytes, and the whole is incubated at 37° C. to convert the fibrinogen into fibrin, and the clear serum is separated by decantation from the contracted coagulum.)

After centrifuging the suspension of leucocytes the denser portion is introduced into Eagle's basal nutrient medium to which 5% by volume of the defibrinated human serum has been added, the quantity of medium being such that the number of leucocytes is 5 million per ml. The suspension thus obtained is introduced into a sealed bottle, so that the liquid occupies from ¼ to ⅛

more particularly about ⅕, of the total available volume. Applicants have found that it is advantageous, to obtain the best final yield of interferon, more particularly to avoid the interferon rising along the bottle wall above the free surface of the liquid, to employ a silicone-coated glass bottle or a polytetrafluoroethylene bottle.

The product is then incubated on the water bath at 37° C. with agitation for about 24 hours, and the suspension is then centrifuged and the supernatant liquid is separated and brought to a pH of 7.3 to 7.4. For this purpose, it is advantageous to adopt the following procedure. Normal hydrochloric acid is added to the clarified liquid to lower the pH value to 2. The liquid is maintained for about 24 hours at about 4° C. Sodium hydroxide is added to bring the pH to 7.3. The product is subjected to prolonged ultracentrifuging (for about 24 hours) and filtered, preferably through a membrane having pores of the order of half a micron, and the pH of the liquid is brought to a value of 7.3 to 7.4 with hydrochloric acid or sodium hydroxide as necessary.

The interferon-containing preparation thus produced be aseptically distributed in ampoules, for example of 5 or 10 ml., and kept at low temperature, for example −80° C.

When cells in organized structures, e.g., amniotic membrane, are employed as starting material, the procedure may be modified as follows.

The amniotic membrane is separated from the chorion at most 6 hours after the expulsion of the placenta. For this purpose the following procedure is preferably adopted. The placenta is hung by means of the umbilical cord, so that it hangs freely, and the amniotic membrane is cut into shreds with surgical instruments. The amnion is washed 4 times with physiological saline until the contaminating matters, more particularly blood clots, mucus and chlorionic particles have been eliminated, the membrane is then macerated for a period varying from 24 hours to 72 hours in Eagle's basal nutrient medium, with daily renewal of this medium.

The macerated membrane is transferred into a 1-litre Fourneau flask and immersed in allantoic liquid containing Sendai virus, irradiated with ultra-violet rays (for example at 5,000 to 6,000 ergs per mm.^{2}), in a concentration of about 10^9 times the LD_{50} per ml. From 150 to 200 ml. of allantoic liquid is employed for a membrane having an average weight of 40 to 50 g. The incubation takes place at 37° C. for 2 hours, with periodical stirring.

The membrane thus infected is preferably dipped into 3 successive baths of physiological saline to eliminate the unadsorbed virus. The product is then macerated for 24 hours in 200 ml. of Eagle's basal medium, to which defibrinated human serum has been added. At the end of this time, the liquid phase is separated and the membrane transferred into 200 ml. of fresh medium, to supply a second crop of interferon. A third cycle may be carried out under the same conditions. The three crops of liquid are mixed and centrifuged at about 4° C.

This liquid mixture may then be acidified with hydrochloric acid and neutralized with sodium hydroxide and then ultracentrifuged and filtered, with final correction if necessary of the pH to a value of 7.3 to 7.4, as already described.

It is also possible to use as starting material human embryos extracted by curettage. Such embryos are trypsinated by the conventional method. The cells are cultivated for a period of one to two weeks in Roux dishes. They may then be subjected to subculture in cylindrical 2-litre bottles, with the aid of a "roller" (i.e., a wheel provided with recesses in which the bottles can roll while remaining horizontal). This cellular culture is then used in the production of interferon in the same way as that already described for amniotic membrane cells.

As already mentioned, the virus used as induction agent may be irradiated before use. It has been found that the production of interferon varies with the degree of irradiation of the induction virus and that, in addition, with certain cells, the irradiation leads to increased production of interferon cells, and in the case of other cells, to reduced production.

To irradiate the Sendai strain virus, the following procedure may be adopted. The viral suspension in allantoic liquid is introduced into a petri dish in such manner that the layer of liquid does not exceed half a centimetre in thickness. During the irradiation, the petri dish is gently agitated with an agitator of the Khan type. The source of ultra-violet rays employed is a "General Electric" germicidal lamp which produces 80 ergs mm.^{2}/sec. at a distance of 28 cm. It has been found that the optimum irradiation is 5,100 ergs/mm.^{2} with Sendai strain virus which is to be used to infect amniotic membrane cells. The production of interferon by the latter is then increased. On the other hand, if the irradiated virus is used with leucocytes, interferon production is reduced.

To determine the dose of irradiation to be used with a given induction virus, and type of infected cell a curve representing the response in quantity of interferon produced as a function of the irradiation dose may first be prepared, and the optimum irradiation dose deduced therefrom.

The invention also provides a process for purifying human interferon on an industrial scale. This process comprises absorbing the interferon on a weekly acidic cation-exchange material buffered to a pH of about 5.9, the material is then washed free of protein with buffers having a pH from 5.9 to 7.0, and the interferon is then removed from the said material by washing with a buffer having a pH of 7 to 8.5. The cation-exchange material is preferably carboxymethyl-Sephadex (Sephadex is a dextran polymer). It is difficult to apply column chromatography on an industrial scale in the case of interferon. The two essential disadvantages are the slowness of the process and the dilution which the interferon must undergo during the elution. These two difficulties are overcome by using bath chromatography. The advantages reside in the rapidity, the high yield and the ready adaption of the method to any volume of solution. While column chromatography is limited by the close relationship between the volume of the specimen and the volume of the resin, by the necessity for slow elution and by the dilution of the biological activity of the product, bath chromatography obviates these disadvantages, while ensuring partial purification (at most 30 to 40 times).

Interferon derived from leucocytes may be purified as follows. Carboxymethyl-Sephadex (C_{50}) is balanced by stirring with 40 to 50 times its weight of 0.01 M phosphate buffer (buffer No. 1) and this is repeated with fresh buffer as many times as is necessary for the pH to be stabilised at 5.9. The crude interferon-containing preparation is dialysed against 30 times its volume of buffer No. 1. The preparation and the treated cation-exchange material are mixed in a polyethylene receptacle rather than glass on which the interferon has more tendency to become fixed) in a proportion of 4:1 by volume. The whole is then agitated for 18 hours at +4° C. To eliminate the unfixed proteins, the gel is centrifuged at 6,000 r.p.m. About 95% to 97% of the interferon is fixed on the cation-exchange material, which is then washed successively with the following buffers: buffer No. 2 containing 0.01 M phosphate, 0.05 M NaCl, at a pH of 6; and buffer No. 3 containing 0.01 M phosphate, 0.075 M NaCl, at a pH of 6.2. After the unfixed or weakly fixed proteins have been eliminated by repeated washing, the fixed interferon is displaced with the aid of the following buffers, used successively: Buffer No. 4 containing 0.01 M phosphate. 0.01 M NaCl, at a pH of 7.5; and buffer No. 5 containing 0.01 M phosphate, 0.1 M NaCl, at a pH of 8. (Buffer No. 2 may be prepared by dissolving 0.01 gramme-molecule of NaH_2PO_4 in 900 ml. of water adding 5 N sodium hydroxide solution to bring the pH to 6, dissolving 0.05 gramme-molecule at NaCl in the

solution and finally adding water to a final volume of 1000 ml. The other buffers may be prepared similarly.)

The repeated washing of the resin and the elution of the interferon are carried out by agitation followed by centrifuging. The results of a series of typical experiments show that this process effects a substantial purification, with an increase of thirty to forty times the specific activity per unit volume of the preparation. This method has again been tested with similar results with volumes from 25 ml. to 1 litre.

The preparations produced as described above satisfy the tests for establishing the presence of interferons. Thus:

(1) They have no direct action on the virus of vesicular stomatitis;

(2) They do not sediment after ultracentrifuging at 40,000 r.p.m. for 4 hours;

(3) They are destroyed by trypsin and resist treatments with ribonuclease and with desoxyribonuclease;

(4) They withstand on the one hand a pH value of 2 and on the other hand a pH value of 10;

(5) They are specific for the cellular species in which they have been produced.

The substance or substances of the interferon type prepared from amnions by the process of this invention have a molecular weight of the order of 160,000, while the substance or substances of the same type derived from leucocytes have a molecular weight of about 25,000.

We claim:

1. Process for the manufacture of a preparation rich in interferon which protect human species of cells and the cellular species in which it has been produced against viral infections thereof which comprises incubating live organized human cells of human placental adnexa including amniotic membranes, chorions, and placentas with a viral induction agent, separating the induction agent not fixed in the said cells, continuing the incubation of the infected cells, separating the interferon-containing supernatant liquid and adjusting the pH thereof to 6.9 and 7.6.

2. Process according to claim 1 in which the viral induction agent is para-influenza type I virus, Sendai strain.

3. Process according to claim 2 in which the virus is irradiated before use with ultra-violet rays.

4. Process according to claim 2 in which the virus is irradiated before use with 5100 ergs/mm.^{2} of ultra-violet rays.

5. Process according to claim 1 in which the organized human cells are amniotic membrane cells which are infected by immersion in a liquid containing the induction agent, washed free of induction agent, and then macerated and incubated in Eagle's basal nutritive medium containing defibrinated human serum.

6. Process according to claim 1 in which, after the incubation, the supernatant liquid is separated by centrifuging followed by the steps of adjusting the pH thereof to about 2, keeping same at about 4° C. for about 24 hours, adjusting the pH thereof to 7.3 to 7.4, and finally centrifuging and filtering to give an interferon-containing liquid.

7. Process according to claim 1 which comprises the further steps of absorbing the interferon on a weakly acidic cation-exchange material buffered to a pH of about 5.9, washing the material free of protein with buffers having a pH from 5.9 to 7.0, and removing the interferon from the said material by washing with a buffer having a pH of 7 to 8.5.

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