Substitution of Milk for Serum in the Production of Human Leukocyte Interferon

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The presence of serum in suspensions of Sendai-induced human leukocytes is necessary for the synthesis of significant amounts of interferon. Very little interferon is obtained from serum-free suspensions. Cow’s milk or milk casein can substitute for serum in the production of high yields of human leukocyte interferon.

Suspensions of human leukocytes require high serum concentrations to yield substantial quantities of interferon (1, 7). Because it is often undesirable to have interferon preparations contaminated with the complex and ill-defined components of serum, attempts have been made to prepare interferon in the absence of serum. Strander (5) reported that a combination of serum albumin and a high concentration of a bipolar ionic buffer such as N-tris(hydroxymethyl)methylglycine (Tricine) can be used as a substitute for serum in the production of potent preparations of human leukocyte interferon. In the present report, the use of milk or milk casein as a serum substitute is described.

MATERIALS AND METHODS

Viruses. The Sendai strain of parainfluenza 1 virus was grown in chick embryos, and the Indiana strain of vesicular stomatitis virus (VSV) was passed in U cells. Details of the procedures have been described previously (6, 7).

Cells. A continuous line, “U,” of human amnion cells was grown in Roux bottles. Leukocytes were stored at 4°C in the presence of 0.5% (w/v) ethylenediaminetetraacetate and purified by treatment with NH4Cl by using a procedure which has been described previously in detail (6, 7). No serum was used during the purification.

Interferon production. Purified leukocytes were suspended at a concentration of 10^9 cells per ml in Eagle’s minimal essential medium (MEM) buffered with 3 mg of Tricine per ml instead of phosphate. The cell suspensions were supplemented with 5% human serum (inactivated by heating at 56°C for 30 min) or with one of the other additives, and volumes of 100 to 400 ml in 2,000-ml round flasks were incubated in water baths at 37.5°C. The flasks were closed with loose foil covers, and the cells were kept in suspension by means of magnetic stirrers. Interferon production was induced by the addition of a priming dose of 100

1 units of human leukocyte interferon per ml, followed 1 hr later by the addition of 300 hemagglutinating units of Sendai virus per ml of suspension. Samples of the suspensions were stored at 4°C before assay.

Interferon assay. Cells were removed by centrifugation, and the supernatant fluids were dialyzed against glycerine-HCl buffer (pH 2) to destroy remaining virus. After back-dialysis to pH 7.3, the interfering activity in each preparation relative to that in the research standard for human interferon was estimated by plaque reduction of VSV in U-cell cultures. The results of all assays are given in terms of the unit which has been assigned to the research standard preparation 67/87 (3).

Milk. Cow’s milk was purchased locally and sterilized by autoclaving at 115°C (0.72 kg per cm²) for 15 min. The color of the milk became light brown during the autoclaving, but no precipitate was formed. The pH was adjusted to 7.3 before use by the addition of 1 N NaOH.

Casein was precipitated from milk by acidification with dilute HCl to pH 4.6 or was sedimented by centrifugation for 90 min at 80,000 × g (Spinco 40 rotor). The resultant casein pellet was dispersed in a volume of MEM equal to the original volume of milk from which the pellet was obtained. In what follows, this suspension has been termed “casein preparation.” The supernatant solution, called whey, remaining after centrifugation was used without further treatment or, following the acid-precipitation procedure, after a readjustment of the pH to 7.3.

Protein concentrations were measured with both the biuret and Lowry methods with each sample, and, since the results were in good agreement in all cases, the values given are the average of the two numbers.

Other materials. Solutions of 20% human albumin, suitable for injection, were prepared by the Finnish Red Cross Blood Transfusion Service. Tricine was purchased from Calbiochem, Los Angeles, Calif.

RESULTS

Serum substitutes. Although many substances have been tested as possible substitutes for serum in interferon production by human leukocytes, in previous studies only ascitic fluid and serum
albumin were found to be active in this respect, and the latter required a high concentration of a dipolar ionic buffer such as Tricine to give titers of interferon approaching those obtained in suspensions containing serum (5). It has now been found that the addition of milk to serum-free leukocyte suspensions consistently results in the production of high yields of interferon upon induction with Sendai virus.

Skim milk (0.05% fat) is as effective as partly skimmed (2.5% fat) or whole milk (3.9% fat); thus, it was used throughout the study. Sterilization of milk by autoclaving does not decrease its activity.

Concentrations of milk in the 10 to 20% range gave optimal yields of interferon (Fig. 1). In comparison, the minimum concentration of serum which can be used to obtain consistently optimal yields is 5% (unpublished data). The stimulating effects of serum and milk are not additive. In a typical experiment, interferon yields of 10\(^{14}\), 10\(^{10}\), and 10\(^{11}\) units per ml were obtained in suspensions containing 5% serum, 10% milk, and 5% serum plus 10% milk, respectively.

**Milk fractions.** As a preliminary step in determining the active principle in milk, skim milk was separated into two fractions by either high-speed centrifugation or acid precipitation of the casein component. The whey and casein preparations were both tested in leukocyte suspensions.

Whey, added in concentrations ranging from 5 to 45% (v/v), stimulated interferon production by a factor of two to five above that in unsupplemented suspensions. Yields of interferon did not differ significantly at different whey concentrations in this range and were all approximately 10 times less than those obtained in the presence of 10% milk. The addition of casein preparations resulted in the production of as much as 10 times as much interferon as the whey when added in concentrations covering the same range. Optimal yields were obtained by the use of 10 to 20% of the casein preparation (Fig. 2). It should be emphasized that the concentrations given with respect to casein refer, in all cases, to the amounts of the casein preparation, prepared as described above, which were added to the leukocyte suspensions. No differences in activity between casein preparations obtained by the centrifugation or the acid precipitation methods were observed.

Concentrations of protein in the casein preparations were determined and were found to range from 15 to 20 g/liter in different preparations. The protein concentrations of milk and human serum, determined at the same time, were 30 and 70 g/liter, respectively. Thus, the total amounts of protein added to leukocyte suspensions by the addition of 5% serum, 10% milk, or 20% of the casein preparation are roughly the same.

**Comparative effects of serum substitutes.** The results of many experiments were pooled, and the data are given in Table 1. It is clear that milk and casein, at optimal concentrations, are almost as effective as serum in stimulating interferon production and are considerably better than albumin as serum substitutes. A small, but significant, amount of interferon is produced in the absence of any of these supplements. With respect to the low activity of albumin, we found that increasing the concentration of Tricine above 3 mg/ml did not increase the effectiveness of albumin as a serum substitute, as has been reported by Strander (5). The activity of albumin in the presence of 3

![Fig. 1. Effect of the addition of different amounts of milk to serum-free human leukocyte suspensions on the concentration of interferon present in these suspensions at 20 hr after induction with Sendai virus.](image1)

![Fig. 2. Effect of the addition of different amounts of a casein preparation to serum-free human leukocyte suspensions on the concentration of interferon at 20 hr after induction. Details regarding the casein preparation are given in the Materials and Methods.](image2)
mg of Tricine per ml in both studies, however, is roughly equivalent. The reasons for the inconsistent results obtained when the buffer concentrations were increased cannot be explained.

**Kinetics of interferon production.** Samples were removed from leukocyte suspensions at intervals after induction with Sendai virus and assayed for interferon production. The results of three experiments have been pooled to give the results shown in Fig. 3; it can be seen that interferon synthesis takes place over the same period of time in all cases.

**DISCUSSION**

The use of milk or casein in the preparation of human leukocyte interferon has some advantages over the use of serum. Not only is it much more economical for mass production of interferon, but the product is also likely to be more suitable for certain purposes. For example, although the use of milk or casein does not result in the production of a crude product with a clearly higher activity per milligram of total protein, the use of casein may simplify the purification of the interferon protein. Also, human serum contains viral antibodies; thus the use of casein could avoid possible problems associated with the presence of these antibodies in interferon preparations.

It seems evident that most, if not all, of the activity of milk resides in the casein fraction. The small amount of activity detected in the whey could result from either lactalbumin or some other whey proteins or from some casein which was not removed from the whey during the fractionation procedures used in this study.

At present, it is not possible to explain how casein can substitute for serum so effectively, especially since so little is known about the active components of serum and the mechanism of serum action is not understood (2, 7). Stranger could only conclude that the serum albumin molecule, or something firmly attached to it, was in all likelihood the most critical of the required serum components but that it alone could not account for all of the activity of serum (7). It is intriguing that a protein like casein, which is chemically and biologically different from all of the proteins present in serum (4), possesses full activity in stimulating human leukocytes to produce interferon. It is, however, well known that both albumin and casein act as carrier proteins for a variety of other molecules. It might be that one such bound molecule is, in fact, responsible for stimulating interferon synthesis in both cases. Both the fact that the effects of milk and serum are not additive and the observation that the kinetics of the production are the same in the presence of serum and milk or casein are compatible with the premise that the same mechanism is involved in each case. Further information on the stimulation of interferon production in Sendai-induced human leukocytes may be obtained by the use of purified casein components.

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### Table 1. Interferon production in the presence of serum or serum substitutes

| Additive       | Conc (%) | Log₁₀ interferon units/ml² |
|----------------|----------|-----------------------------|
| Human serum... | 5 (v/v)  | 4.17 ± 0.06                 |
| Human albumin..| 1 (w/v)  | 3.04 ± 0.07                 |
| Milk .........  | 10 (v/v) | 4.04 ± 0.05                 |
| 20 (v/v)       | 4.07 ± 0.06 |
| Casein prepn...| 10 (v/v) | 3.70 ± 0.19                 |
| 20 (v/v)       | 3.84 ± 0.19 |
| Whey ...........| 5-45 (v/v) | 3.13 ± 0.18 |
| None ...........| 2.43 ± 0.09 |

* Each value is the average interferon titer and the standard error calculated from the results of 5 to 40 separate experiments.

**Fig. 3. Kinetics of interferon production in Sendai-induced human leukocyte suspensions containing 5% human serum (○), 10% milk (●), 20% casein preparation (□), and 1% human albumin (■) or in unsupplemented suspensions (△).**
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