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SUMMARY OF PROCEEDINGS OF A WORKSHOP ON SERUM FOR TISSUE CULTURE PURPOSES*

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A Workshop on Serum for Tissue Culture Purposes was held at the W. Alton Jones Cell Science Center, Lake Placid, N. Y., April 29–30, 1971. The workshop was comprised of 50 to 60 individuals representing both serum users and serum distributors. The basic aims of the workshop, as stated by Dr. Sergey Fedoroff in his welcoming remarks, were to consider, in free and frank discussion, those problems which face investigators and commercial producers; to formulate plans to deal with these problems; and to set priorities for action. Hope was expressed that the TCA Committee on Sera (Dr. A. J. Kniazeff, Chairman) might assist in the planning and coordination of a program to improve serum quality and to set up serum standards to serve as guidelines in quality control. The program of the workshop follows.

April 29, 1971

Welcome: President, Tissue Culture Association

Session 1:

	Speaker
Chairman: H. E. Hopps	
Contamination of sera with mycoplasma.	M. F. Barile
Contamination of sera with viruses, bacteria, and fungi.	A. J. Kniazeff
Panel Discussion: Sterilization of sera.	G. A. LoGrippo C. F. Neilson
General Discussion:	
Sterility at the source.	
Sterility testing.	
Effects of bacterial by-products.	

Session 2:

Chairman: C. Waymouth	
Biochemical and biological tests of sera.	
Quality control.	C. W. Boone
General Discussion:	
Composition of sera.	
Effect of sera on cells in cultures.	

April 30, 1971

Session 3:

Chairman: K. K. Sanford	
Sources, maintenance, and general problems related to animals.	D. L. T. Smith
General Discussion:	
Problems of procurement of serum.	
Future prospects for improvement of serum.	
Serum fractions.	
Chemically defined medium.	
Summary of the Workshop and future plans.	L. L. Coriell J. T. Duff

MICROBIAL CONTAMINATION

Mycoplasmas. Dr. M. F. Barile (DBS) reviewed the published literature and his own experience and reported that 0 to 4% of primary cultures were contaminated with Mycoplasmas (average 1%). Continuous cell cultures were contaminated with Mycoplasmas in 50 to 92% of cases. There were positive correlations between Mycoplasma contamination, the use of antibiotics, and the use of serum in the culture media. Seventy-two percent of cell cultures with antibiotics were contaminated, whereas only 7%

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of cultures without antibiotics were contaminated. Fifty-six percent of cultures containing sera were contaminated, while in cultures where no serum was used, no Mycoplasma contamination was detected. There appear to be cycles of Mycoplasma contamination, particular species predominating at various times. Human strains have accounted for 56% of reported contaminations, swine for 11%, bovine for 29%, and miscellaneous species for 4%. During the past 2 years, bovine strains (*Mycoplasma arginini* and *Acholeplasma laidlawii*) have made up a large proportion of reported contaminations. Dr. Barile presented data (to be published in the Proceedings of the Society for Experimental Biology and Medicine, November, 1971) concerning the first successful isolation of Mycoplasmas from commercial bovine sera. The data indicated that contaminated bovine serum is the major source of bovine Mycoplasma contamination of cell cultures.

Success in isolating Mycoplasmas from serum is associated with the volume of serum employed in testing. Twenty-five milliliters or more of serum per 100 ml of broth medium should be used. This change in sampling technique results in a striking increase in the number of positive Mycoplasma isolates. A total of 379 lots of sera were examined, including 239 unprocessed sublots of bovine sera (184 fetal and 55 calf) and 140 final processed lots of fetal bovine sera. Mycoplasmas were isolated from 55 of the 239 unprocessed lots (23%) while 18 of the 140 final lots (12.8%) yielded positive cultures. Of 13 strains examined, 11 were identified as *M. arginini*. Two strains may represent new species. Dr. Barile reported that if the horse serum component was deleted from the broth medium, and if the cultures were incubated at 32°C with weekly subcultures to agar medium, *A. laidlawii* could also be isolated in some final lots of fetal bovine sera.

The need for large volume testing was stressed. An inoculum of 0.1 to 0.5 ml might be suitable for detecting massive infection but not the low levels of organisms often present in bovine sera.

From the open discussion, it became evident that several laboratories have, in the face of unreliable safeguards against contaminated sera, adopted various empirical procedures to protect their cell cultures from infection with organisms that are hard to detect in serum. Dr. Fogh refilters all serum used in his laboratory. The sera are first filtered through a 1.2-μ Millipore filter, then through a 0.45-μ Millipore filter, and finally through a 0.3-μ Millipore filter. Mrs. H. Hopps and Dr. Uhlendorf have both resorted to refiltering of the final complete medium. Dr. Coriell heats all sera to 60°C for 30 min. Each of these laboratories has been singularly free of accidental mycoplasmal contamination of cell cultures. No doubt they all sacrifice some growth-promoting factors to achieve this freedom from contamination.

Viruses. Dr. A. J. Kniazeff reviewed the types of viruses that have been isolated from pools of fetal bovine serum collected by sterile and nonsterile procedures. Identified viruses include the bovine Herpesvirus, bovine virus diarrhea, parainfluenza 3, and bovine enteroviruses. Several isolates have not yet been characterized. Taking into account the experiences in other species with reference to the common presence of syncytial viruses, it is quite likely that among the uncharacterized agents the bovine syncytial virus may be found. These isolates were made during studies conducted at Hyland Laboratories with Dr. Charles Molander (now with the KAM Laboratories in Grandview, Mo.) and reported in this issue of IN VITRO. Of the 150 lots of bovine serum tested, viruses were isolated from 18 lots, that is, 12% of the pools tested. It is well established that all four identified viruses invade the fetus and produce viremia. Since some of the isolates were made directly from bovine serum collected aseptically, it is almost certain that, in the majority of cases, the contamination of the serum with these viruses is attributable to the presence of the agents in fetal blood and is not a result of external contamination.

The incidence of viral contamination of serum appears to be dependent on the age of animal from which the blood is drawn. Thus it was noted that viruses were infrequently isolated from newborn and agamma serum; none was yet isolated from sera of older calves; and a very small percentage of isolations was made from bovine sera of adult animals.

The procedures of virus isolation from bovine serum have been described, and several companies distributing the product perform tests for the presence of bovine Herpesvirus, bovine virus diarrhea, and parainfluenza. The usefulness of such tests for an investigator depends, to a cer-

tain degree, on the intended application of the serum. There is no question that, for research studies or vaccine production with bovine viruses, such tests are quite useful. For propagation of human cell lines, the hazards are not defined; and, although it has been demonstrated that in experimental exposures bovine Herpesvirus may infect human cells, there are no data on the incidence of such infections, if any, in the course of the usual human or nonbovine cell propagations. Until definitive studies are carried out to determine the infectiveness of these viruses to nonbovine cell lines and the consequence of such infections, the investigator must decide how seriously he should consider such viral contaminants.

Dr. Coriell revealed that in his laboratory electron microscopic examination of sedimented pellets from bovine sera showed virus-like particles. Most of these "viruses" do not appear to grow in human cells, so they are not detected in culture. On the basis of these observations, Dr. Coriell questions the general practicability of monitoring bovine sera for the presence of viruses.

The incidence of bacterial contamination in sera that are not collected aseptically is high, so it is possible that phage infections may develop in such sera. There is no information on the subject and any discussion could only be speculative.

Bacteria and fungi. Data concerning bacterial and fungal contamination are reported in the accompanying paper by Boone.

Sterilization of Sera and Sterility Testing

Several methods for sterilization of bulk lots of sera were described, in addition to the time-honored procedures of filtration through glass, Selas, Seitz, or membrane filters.

Dr. G. A. LoGrippo described a method of combined ultraviolet irradiation and β-propiolactone treatment of serum. The method is reported to be effective but is not licensed in the United States because β-propiolactone may have carcinogenic properties.

Dr. C. F. Neilson, of Gray Industries, Inc., described their method of serum sterilization with nonionizing radiation. The method is said to permit sterilization of glass-packaged serum without alteration of serum proteins. Some participants felt that it would be desirable for Gray Industries to provide to users more detailed information on the methods of production, of sterility testing, and on the growth-promoting capacity of their product.

A procedure of sterilization by means of high energy electrons was also briefly discussed. Data were presented demonstrating that complete inactivation of even the smallest picornaviruses can be achieved. However, the levels of energy necessary to achieve complete sterility produce obvious alterations in serum proteins. Studies of this method are continuing.

For determining the sterility of products, it was recommended that a spectrum of five or six different test media be used, to insure the isolation of fastidious microbial contaminants. The test media should be incubated both at 30 and 37°C. Tests for mycoplasmal contamination should be carried out in accordance with the procedures recommended by Dr. Barile.

Consideration of Some of the Major Factors Influencing the Usefulness of Serum in Cell Propagation

Dr. Charles Boone reported the results of a very extensive fetal calf serum study which was conducted at Hyland Laboratories. His paper appears in this issue of In Vitro.

Many users of fetal bovine serum believe that poor quality in fetal bovine serum can be attributed to improper handling during the original procurement of blood. Dr. D. L. T. Smith pointed out that nearly all serum is obtained from animals killed at slaughterhouses, which are highly unsuitable places for collection. Animals are usually brought to the processing plants a week or 10 days before slaughter, are frequently transported long distances, and are thrown together with other animals under conditions of restricted space and limited food and water. These factors lead to stress and increase the probability of outbreaks of communicable disease which may reach epizootic proportions. Such syndromes are commonly referred to as "shipping fever." Under such conditions of stress and infection, the total physiological state of the animal is affected, including the composition of the serum. A result may be serum of low quality. Several suggestions were made, to eliminate these undesirable conditions of serum collection. It was suggested that pathogen-free or even germ-free herds could be established and used for serum collection. A simpler, but possibly

effective, procedure would be the isolation of healthy animals in fenced-off areas. These procedures would permit the collection of satisfactory serum. All such methods would involve very heavy expenditures in construction and in animal husbandry, making them impractical for commercial organizations.

To complement this discussion, several representatives of serum suppliers pointed out that, in the majority of cases, blood is collected under the best conditions which can be achieved in the slaughterhouse environment. They also pointed out that, in many instances, they cannot place their own people in the slaughterhouses to apply more hygienic procedures, because they cannot get the necessary permission form the packers. However, Hyland Company in the past, and Armour Company at present, obtain fetal calf serum in accordance with the procedures which were used by Hyland in the production of the "Special Fetal Calf Serum." Representatives from both companies indicated that the cost of such specially processed serum is considerably higher than normally processed serum. Dr. Charles Boone, who acted as a project officer on the "Special Serum" study at Hyland Laboratories, indicated that, although the contractor was paid $85.00 per liter of this special serum by National Cancer Institute, the company lost money.

Some of the company representatives, as well as some investigators, expressed a strong opinion that the fetal bovine serum now being supplied by most of the companies is, in truth, considerably better than many investigators think it is. In their view, further measures to improve the product would result in considerable increase in cost, probably without any commensurate improvement in the cell-supporting qualities of the product. The major difference between the two types of serum in the Hyland study (Boone) was in the incidence of microbial contamination, and not in cell growth-promoting capacities.

Alternate Approaches to Serum Procurement and Processing

Some consideration was given to more sophisticated methods of serum procurement. A suggestion which merits investigation is the establishment of high grade herds which would be maintained on special diets and subjected to periodic health examinations. Blood collection and serum harvesting in such establishments could be carried out under the most desirable conditions, thus obviating the inadequacies inherent in slaughterhouse collection. In such an operation red blood cells could be returned to the animal, and the frequency of bleedings increased considerably without upsetting the normal physiological state of the animal. Depending on the results of comparative studies aimed at determining the relative values of sera procured from animals of various age groups, such an operation could process serum from young calves, baby beef, or adult cattle. Should it appear advantageous, animals which reached a certain age could be sold off. A higher grade of serum could be produced under such conditions.

Aspects of Serum Composition

Serum composition, the state of its components, and the alterations which serum undergoes during processing and storage play a large part in determining the suitability of serum for cell culture purposes. These important subjects were summarized by Dr. Richard Holmes. He pointed out that serum consists of a vast array of substances, including amino acids, carbohydrates, lipids, nucleotides, hormones, vitamins, enzymes, and proteins. It contains a full complement of intermediate metabolites originating from the anabolic and catabolic activity of all the cells in the intact animal. It must be considered as a dynamic fluid, in balance with all the various cellular functions. The moment blood is drawn and serum is prepared, its composition progressively changes, largely as a result of continuing enzymatic activity. Each individual serum is qualitatively and quantitatively an entity. Species and age influence its composition, particularly the proteins, such as globulins, lipoproteins, glycoproteins, and immunoglobulins. The protein composition of fetal sera is generally less complex than that of adult sera. Diet, health, genetic characteristics, and species affect serum composition. Over 200 proteins have been identified in human serum, some in large amounts, and numerous enzymes in small amounts. Many small molecules become attached to proteins, and these complexes may dissociate, sometimes with difficulty. Serum has high reducing properties, and oxidation during preparation may deleteriously affect both large and small molecular weight components. It has

been suggested that serum, though more properly plasma, is a macromolecular structure with the component parts (particularly the proteins) associating through salt linkage, hydrogen bonding, and hydrophobic adsorption. There is a growing body of experimental evidence to support this idea. Identification of individual proteins, or prosthetic groups carried by many proteins, may make it possible to devise new chemically defined media that do not require the addition of serum.

Storage of Serum

Storage conditions affect the quality of serum more than is sometimes realized. Deterioration during storage can occur from enzymatic activity. Oxidation, during both preparation and storage, inactivates many vitamins, including ascorbic acid, vitamins A and D, and many flavins. The methods used to freeze or thaw, if serum is frozen, can cause aggregation of chylomicrons, lipoproteins, and cold-precipitable globulins. These substances vary greatly in quantity, depending upon the species, health, and age of the donor. Both lipids and globulins are generally lower in fetal than in adult sera. Sterile sera, high in low density lipoproteins, readily aggregate on incubation. The resultant precipitates float to the top in density gradients. In storage studies where the amino acids were determined quantitatively, progressively lesser amounts of amino acids were released as the temperature was lowered. There is some increase in total amino acids even when the serum is stored at −20°C. A stable condition is attained only with sera stored at −70°C. The problem of oxidation is similar to that experienced with various foods and oils. The possible addition of antioxidants would have to be approached with caution. Storage at −70°C prevents oxidation. Methods for measuring the reducing capacity of serum have been published.

Comparative Values of Bovine Sera Collected from Animals of Different Ages

Dr. Kniazeff discussed the use of sera from cattle of different ages. There are four categories of serum: fetal bovine serum, collected from unborn fetuses; newborn calf serum, collected at birth or within a few weeks of parturition; baby beef serum, taken between 4 and 6 months of age; and adult bovine serum for which, so far, no age limit has been specified.

Newborn calf serum, particularly when it is collected within the 1st week post-partum, is presumed to have many of the growth-promoting characteristics of fetal bovine serum. Unless the newborn calf is deprived of colostrum, such serum will have high levels of antibodies. In the early stages of its life, the newborn calf is protected by maternal antibodies from various types of infections. Consequently the serum of such animals is likely to be free of endogenous microbial contaminants. Such serum has been used successfully by many investigators. It has been found to be suitable for cell cloning and plating, and for other demanding procedures. In the past, the procurement of such serum in large volumes was not difficult. Presently, however, because of the high cost of beef, the price for male calves is quite high. It would require an economic evaluation to determine whether the procurement and distribution of such serum by commercial organizations would be practical.

The term "baby beef" is not applied to a very young calf such as a vealer, but designates young steers which are slaughtered in certain parts of the country. Without knowing how widespread is the slaughter of such animals at present, it is difficult to estimate how much serum could be procured from such sources. Several individuals working in Florida have had considerable experience with this type of serum. The product was found to be satisfactory for routine propagation of both primary and established cultures. Its use for cloning procedures, to our knowledge, has not been evaluated. Since these animals are slaughtered at a time in life when maternal antibodies are exhausted, viruses may invade the blood stream and lead to viral contamination of the serum.

Adult bovine serum, procured from animals above 1 year of age, has been used in several laboratories. The serum was found to be reasonably satisfactory for the propagation of primary cultures and for some established cell lines. When large harvests of serum were made from a single animal, considerable variation in results occurred when the serum of another animal was substituted. Sera pooled from a larger number of animals would presumably reduce the degree of variability. The product was not found to be

generally satisfactory for the more demanding procedures of cell cultivation.

Development of Serum-free Media

As an alternative to bovine serum in culture media, Dr. Hayflick suggested that attention be given to the use of ultrafiltrates. Dr. Pumper reported that Bactopeptone (Difco), which can be autoclaved, provides an adequate environment for the growth of many cell lines. Lactalbumin hydrolysate and yeast extract have been used in a similar manner. Dr. McMorine (Connaught Laboratories) indicated that soybean hydrolysate also shows promise as a substitute for serum.

Drs. Waymouth, Evans, Sanford, and Holmes all agreed that work must continue on the development of chemically defined media for cell propagation without a cell adaptation period, i.e. those which are suitable for primary cell cultivation.

Dr. Holmes named several approaches to the problem of growing primary cells in chemically defined medium: (a) devise methods of cell transfer that do not injure the outer cell surface; (b) attempt to control the state of reduction to match that of the original tissue; and (c) isolate key proteins required for the growth of primary cells, with a view to studying their function and duplicating it by the use of small molecules of known structure. It might be useful to follow a program similar to that described by Heremans and Schultz in *Molecular Biology of Human Proteins*. They have well summarized the confusion that exists concerning the biological activity of fetuin as described by Pedersen; Fisher, Sato and Puck; Michl and Mar et al.; and Spiro. Similar confusion is often encountered with the human alpha protein growth factor described by Lieberman and Ove; Holmes; Healy and Parker; and Pirt and Tozer. It would be useful to correlate these functions with the biological activity of peptides described by Waymouth; Merchant; Pumper; Holmes; and Markowitz. Coordination of these functions could lead to identification of the requirements for growth of various primary cells.

Dr. Aaron Freeman stated that everyone agrees that serum sold for use in culture media should be sterile, but that it is difficult to define what is actually meant by sterility. On the practical side, no supplier of serum can guarantee that any single bottle of serum is sterile, because all quality control procedures are based on statistical evaluation. There is, in fact, no agreement as to what constitutes a reasonable sample for sterility tests. Is it 5%, 10%, or higher? There is also no agreement about which contaminating organisms should be considered in sterility tests: e.g. Mycoplasmas, bacteria, fungi, viruses, and phages. If all are to be considered, how should these agents be tested? The need for, usefulness of, and nature of any formal standards for commercial sera will have to be very carefully considered.

The Committee on Sera is interested in the following questions:

1. Should the commercial serum suppliers be encouraged, requested, or required to present a declaration of standards (a) in their catalogues, or (b) on the labels of the products?
2. If so, what characteristics of the sera are so basic that they should be listed?
3. Is each individual lot to be characterized, or may the label merely state the acceptable limits?

Summary (L. Coriell, J. Duff)

The outstanding advances brought out at the conference were: (a) Mycoplasmas are common in fetal bovine serum and can be detected by a new procedure which uses 25 ml rather than 0.5 ml of serum in each test; (b) there seems to be a negative correlation between the presence of free fatty acids in the serum and its ability to promote growth of cells; (c) the special fetal calf serum prepared in the serum testing project provided a superior serum for tissue culture.

Serum is the most unstandardized, variable, and unpredictable of the ingredients that go into tissue culture media. Until some of the problems are solved, this imperfect source of enrichment has to be used for the cultivation of many cells. In the meantime, a number of suggestions may help in avoiding inherent problems. These include the exclusion of antibiotics from the culture medium wherever this is feasible. Since the nutritional requirements of different cells vary, it is important for the investigator to pretest each serum lot for sterility and for ability to support the growth of his cells. Sterility tests should use multiple media, preferably five or six

media, and incubation at two temperatures, e.g. 30° and 37°C, and should include a test for Mycoplasmas using 25 ml of inocula. Commercial production of bovine sera could be improved by following the procedures used for the special fetal calf serum. The objective should be to determine that serum labeled and marketed as sterile is free from bacteria, yeast, fungi, and Mycoplasmas.

Consideration should be given to establishment of a special testing laboratory for evaluation of the sources and procedures used in serum procurement, to guide members of the Tissue Culture Association, and manufacturers if they choose to use it. Scientific journals should require a statement of tests used to exclude contamination when accepting reports of studies with cell cultures.

The serum problem will not be solved until substitutes which can be sterilized by heat have been perfected. It is recommended that research should be supported on the perfection of defined media, on serum substitutes, and on better ways of identifying and/or eliminating alien viruses from serum. The pooling of information and the advances reported for the first time at this Workshop give hope for continued progress. All participants were encouraged that additional progress can be made and that the many problems are capable of solution through further work, and exchange of ideas between user and producer such as took place at the Workshop.