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USE OF A DIPLOID CELL LINE TO DETECT TOXIC COMPONENTS IN MEDICAL IMMUNOBIOLOGICAL PREPARATIONS

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Medical immunobiological preparations (MIBP) is the term used to describe vaccines and sera used for prevention and treatment of infectious diseases [11]. Control methods included in the requirements for biological preparations, although approved by an expert committee of the World Health Organization, are not laid down by law and require continuous updating [10].

The need to revise the safety regulations for MIBP has increased with the publication of new data on the properties of chemicals [9] present in the composition of MIBP and also by the increase in the number of allergic and autoimmune diseases [1, 2, 9].

WHO experts recommend the extensive use of cell cultures as a substitute for experiments on animals, not only on humanitarian and economic grounds, but also to obtain more objective and informative data on the quality of a preparation which may be administered to man [3, 5]. This is particularly important when studying the effects of small doses of substances, whose action on cells and their structural components may not be manifested immediately after administration of the preparation to a whole organism, but not until years later [5, 7, 8].

In the USSR [11] parameters of acute toxicity on animals and production of allergic reactions in man are used to characterize the safety of MIBP. This system for safety control does not give a complete evaluation of the quality of the MIBP.

The anti-Pertussis-Diphtheria-Tetanus vaccine (APDTV), which we used as the model for our original investigations [6, 12], is considered to be more prone to produce reactions than any other biological prophylactic agent used at the present time [2, 13]. Safety of APDTV is determined by giving a single injection of the ready prepared form to guinea pigs in a dose equal to four times the human dose [11]. WHO experts state that methods assessing the quality

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of APDTV have a low level of reproducibility, they are expensive [10], and they need revision and improvement [10, 13].

To study the properties of specific antigens and chemicals and to detect any possible harmful action of these substances in different doses on cells, permanent cell lines of varied origin are widely used [4, 7, 14].

The aim of this investigation was to study the advantages to be gained from including cell cultures in the system of preclinical methods for the control of MIBP.

EXPERIMENTAL METHOD

For the purpose of comparison, permanent cell line FL and diploid line of human embryonic fibroblasts (DKCh), obtained at the Research Institute of Immunology, Academy of Medical Sciences of the USSR, were used in the experiments. The following nutrient growth media were used: for permanent line FL a mixture of medium 199 and Eagle's medium (1:1) and 5% inactivated bovine blood serum (IS); for the DKCh culture — Eagle's medium (obtained from the Research Institute for Poliomyelitis and Virus Encephalitis, Academy of Medical Sciences of the USSR) and 10% IS. The maintenance medium used during the experiments in test tubes was the same for all types of cultures and consisted of a mixture of medium 199 and Eagle's medium (1:1) without serum. No antibiotics were added to the medium for all the experiments with cell cultures.

The procedure of separating the cells from glass consisted of washing the cell monolayer twice with a mixture of versene and trypsin solutions (4:1). The culture was then allowed to stand, with the monolayer underneath, at room temperature for 7-10 min. The cells which separated from the glass in the form of a loose monolayer, were covered with nutrient growth medium. The concentration of cells in one tube containing 1 ml of growth medium for the experiments was $4 \cdot 10^5$ cells for the permanent line and 10^6 cells for DKCh. This is the optimal cell concentration, yielding a culture in tubes suitable for work after 18-24 h.

The character of action of chemicals, the presence of which was "permitted" many years ago, for example, even before introduction of APDTV [10, 11], on cultures of FL and DKCh cells was investigated: formaldehyde 0.02% was used as inactivator, aluminum hydroxide up to 2 mg/ml as the adsorbent, and the mercury salt, merthiolate 100 μg/ml, as the preservative. This quantity of chemicals is contained in 1 ml of APDTV, ready for use, to be injected three times into an infant during the first 6 months of life. The injection is given parenterally. The action of each chemical substance on the cells was studied separately, and its quantity in 1 ml of the ready to use form was taken as the initial or "whole" concentration. Serial double dilutions were prepared in maintenance medium and 1 ml of each dilution was added to a tube; four tubes were used for each dilution. As the control, four tubes containing the same cell culture, grown from the same passage of the mother strain, and in which the growth medium was replaced by maintenance medium (uninoculated cells) [6, 12], were used as the control.

The action of different concentrations of each chemical on viability of the cells was compared in two stages: 1) intravital microscopy of the cells in the tubes — daily visual observation of the state of the monolayer of the treated cultures parallel with the controls. The onset of a cytotoxic action (CTA) was recorded after 24, 48, and 72 h. The final reading of the results was done after 72 h (using the classical 4-+ system), depending on the degree of degeneration of cells in the monolayer: 100% degeneration (++++), 50% (++), beginning of degeneration (+−), and absence of CTA (−); 2) for subsequent more rigorous control of the limit of cell damage (detection of any possible latent damage) resulting from exposure of the culture to small doses of chemicals, the method of determination of the cytotoxic index (CTI) was used. This is used to detect the cytotoxic effect of cellular and humoral antibodies [15]. For this purpose, after the first stage, namely determination of the degree of CTA, when the first concentration of the chemical compound not causing any visible morphological changes was established, the state of the cells was studied in culture treated with this first "nontoxic dose" and also by the next two dilutions. For example, in Tables 1 and 2, for the DKCh culture treated with formaldehyde, this corresponded to dilutions of 1:32, 1:64, and 1:128. The percentage of living and dead cells was determined [15] in cultures treated with "nontoxic doses" (in four tubes with each dilution), and in four control (uninoculated) cultures. The results were analyzed by Hellstrom's method.

TABLE 1. Degree of CTA of Chemicals Contained in 1 ml of an MIBP Ready for Use

Cell culture	Death of cells in corresponding dilution, in percent								
	formaldehyde (0.02%)			aluminum hydroxide (2 mg/ml)			merthiolate (100 µg/ml)		
	100 %	50 %	without CTA	100 %	50 %	without CTA	100 %	50 %	without CTA
FL	1:2	1,4	1:8	1:16	1:32	1:64	1:64	1:128	1:256
DKCh	1:8	1:16	1:32	1:32	1:64	1:128	1:512	1:1024	1:2048

TABLE 2. CTA (CTI) of "Nontoxic" Concentrations of Chemicals

Cell culture	Formaldehyde (0.02 %)		Aluminum hydroxide (2 µg/ml)		Merthiolate (100 µg/ml)	
	Dilution	CTI	Dilution	CTI	Dilution	CTI
FL	1:8	0,28	1:64	0,25	1:256	0,17
	1:16	0,17	1:128	0,18	1:512	0,17
	1:32	0,06	1:256	0,1	1:1024	0,04
Control		0,04				
DKCh	1:32	0,3	1:128	0,2	1:2 048	0,2
	1:64	0,2	1:256	0,15	1:4 096	0,15
	1:128	0,12	1:512	0,1	1:8 192	0,12
	1:256	0,06	1:1024	0,08	1:16 384	0,08
Control		0,08				

EXPERIMENTAL RESULTS

All the chemicals contained in MIBP in "permissible doses," were found to have an irreversible lethal action on FL and DKCh cells in culture. The presence of CTA was noted under the low power of the microscope after several serial dilutions of each substance.

According to views based on quality standards of antiviral chemotherapeutic agents, components of MIBP must not have a cytotoxic action on cells in culture, since they are intended for administration to a healthy contingent of people and, in particular, to children, and not for the purpose of treatment, but for prevention.

The experiments also showed that the DKCh cell culture is a more sensitive test system than the permanent FL line to the toxic effects of the nonantigenic components of MIBP present in their ready for use forms. The series of investigations, like the previous one [6, 12], showed that the degree of CTA, i.e., the subdose causing 50% death of cells in the monolayer (Table 1), determined by the usual method of visual microscopy, cannot be taken as the limit for cell damage in culture. The first dilution, for example of formaldehyde in the DKCh culture (1:32), not giving rise to visible morphological changes, cannot guarantee the absence of cells in a pathological state. To obtain a more complete idea of the limit of cell damage, additional methods must be used: subculture [6, 12] or subculture preceded by determination of CTI (Table 2). By subculturing cells into which critically low concentrations of MIBP or their components have been introduced, it is possible to preserve the damaged culture and subsequently to study its properties with respect to certain parameters, such as sensitivity to viruses or toxins in comparative experiments with the control mother culture, maintained in the laboratory.

Conventionally designating the subculture + MIBP method [6, 12] as test 1 and the CTI method as test 2, the writers recommend the use of test 1 as being more suitable for studying the properties of damaged cells over long periods of observation. Test 2 can be used in everyday practice as a rapid test by which not only can the presence and degree of CTA be established within a short time (72 h), but the action of critically low concentrations of chemicals contained in MIBP can also be detected.

Cell cultures can thus be used as a test for evaluating the safety of MIBP and, in particular, those used in pediatric practice for mass prophylaxis. Cell cultures satisfy all the demands for informativeness which any experimental model must meet. These are, first, a system highly sensitive to the action of very low concentrations of MIBP and their components, and with a high degree of reproducibility, i.e., statistical significance of results reflecting the degree of accuracy. Second, the biological model must be adequate, enabling the toxicity

of substances which are components of MIBP to be studied at the cellular level, demonstrating latent cell damage, which cannot realistically be discovered in the whole organism, and predicting, i.e., stimulation of pathological processes at the cell level. Adequacy and predictability are achieved by including DKCh cell cultures in the control system, for they provide experimental conditions that are the closest possible to those pertaining in the living organism, and enable processes taking place in the cells at the site of parenteral injection of MIBP to be studied.

With the successful completion of scientific research into the control of vaccines and sera by the use of cell cultures it will be possible to replace *in vivo* tests by more reproducible and standard *in vitro* tests.

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