3008
RECEIVED BY DTIC OCT 14 1969

MASTER

Report No. C00-1645-3

Final Technical Report

Contract No. AT (11-1) - 1645

"Mutagenesis of Enteroviruses by Gamma Rays"

1 September 1966 - 31 August 1969

LEGAL NOTICE

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:
A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or
B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.
As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.

Dean O. Cliver, Ph. D.
Principal Investigator
Food Research Institute
University of Wisconsin

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

"Mutagenesis of Enteroviruses by Gamma Rays"

This study has been intended to determine, by laboratory techniques, whether pasteurizing doses ($\leq 500,000$ rad) of irradiation are likely to induce genetic changes of potential public health significance in viruses. The model system comprised four types of enteroviruses in laboratory diluents and a cobalt-60 gamma ray source. Cobalt-60 was selected because it seemed most likely to be used in the irradiation of foods in the near future. The choice of laboratory diluents, rather than foods, as vehicles for virus during irradiation was based upon expediency and the assumption that no bias of the test results would be introduced in this way. Enteroviruses were chosen as the model agents because the epidemiologic record indicated that they were transmitted in foods (1, 2) and because it was supposed that they would be among the least sensitive of the viruses to the inactivating effect of cobalt-60 gamma rays (3). A recent report indicates that the differences in radiation sensitivity of viruses are quite small, D values being of the order of $0.43 \text{ Mrad} \pm 10\%$ (4). However, the availability of laboratory tests for a number of so-called genetic markers of poliovirus still made the choice seem a good one.

Each of the types of mutations sought was thought to have potential public health significance. Laboratory tests were devised or adapted to measure properties of the subject viruses which were thought to reflect virulence, host range, tissue tropism, and antigenic specificity. The attributes cited were all studied in tissue culture. This should have made possible a relatively high order of sensitivity and reproducibility in the tests, but it also means that caution must be used in extrapolating the test results to what might be expected to occur if the host were instead an

entire animal organism.

METHODS

Tissue Cultures. Primary rhesus monkey kidney (PMK) cell cultures comprise the basic system in which virus tests and titrations were performed. Our tissue culture methods are described in detail in a recent publication (5). Primary cultures were also produced from kidneys of adult, market-weight swine (PASK) and of fetuses from approximately 6 weeks' gestation to near-term (PF8K).

Viruses. Four types of human enteroviruses, all of which have been used as experimental agents in our laboratory for some time, served as model viruses in this study. Each had been subcultured from an isolated plaque on three successive occasions in an effort to achieve genetic homogeneity. Poliovirus type 1 (Po-1), strain CHAT, was obtained from the American Type Culture Collection (ATCC) and was said to be an attenuated strain. Some of our results suggest that it may have become altered during laboratory passage. Coxsackie virus type A-9 (CA-9), strain P. B. (Bozek), is a prototype obtained from the ATCC. It was originally isolated from a case of aseptic meningitis. The Coxsackie virus type B-2 (CB-2) strain we used was undesignated but had originally been isolated in a respiratory disease study at Great Lakes Naval Training Station. ECHO virus type 6 (EC-6), strain D'Amori, is a prototype which was obtained from the ATCC, having been isolated originally from a case of aseptic meningitis.

Irradiation. Irradiation services were provided by the Chemistry Department, using their cobalt-60 source. Since we adapted our virus samples to their sample holder, they also were able to provide dosimetry based upon dose delivered per unit time of exposure. Virus suspensions are irradiated as undiluted infectious tissue culture fluid using a volume of 0.2 ml per

6mm x 6" sealed Pyrex glass tube. The initial set of samples received dosages of 0 (controls), 0.25, and 0.5 Mrad. A second set of CA-9 and CB-2 tubes was irradiated subsequently; and, due to an error in calculation, the dosages in this series were 0, 0.19, and 0.38 Mrad. Since these dosages are well within the "radiation pasteurization" range, the viruses were used rather than irradiate another batch and impose further upon the chemists.

Tests. Two of the selected genetic markers were said to be correlated with virulence. Each attempts to measure, in tissue culture, the ability of a virus to replicate under adverse conditions which might occur during the inflammatory response of infected tissue in the whole host organism. The RCT/40 (reproductive capacity at a temperature of 40C) marker assesses the effect of elevated incubation temperature upon virus replication. A simple test for this marker involved limited thermal exposure (LTE): synchronously infected cultures were incubated for the first 18 hr at 40C and thereafter at 36-37C (6). Results were expressed as the ratio of plaque counts in the LTE flasks to those in comparable flasks incubated continuously at 36-37C. It was soon found that the ratios obtained were sufficiently large to characterize a defined virus strain as RCT/40⁺ or RCT/40⁻, but that the formation of a plaque after LTE treatment of an RCT/40⁻ strain was not a sufficiently unique event to permit its use in selecting presumptive mutants. We therefore adopted continuous incubation at 40C as the challenge condition (7). Ratios of the order of 10⁻⁶ are not uncommon for RCT/40⁻ strains tested in this way, so the probable significance of any plaque formed at 40C from an RCT/40⁻ strain was greatly enhanced.

The other "virulence" marker, d, relates to the ability of a virus to replicate in an acid environment such as, it is said, might also occur

during a local inflammatory response in infected tissue. Acid conditions are imposed by reducing the level of NaHCO_3 added to the agar overlay (7). Results are scored as the ratio of plaque counts under acid overlay to those under standard overlay (36-37C incubation) and have presented the same problems of interpretation as were encountered with the LTE version of the RCT/40 test. In some instances, a "factorial" design was used in which four sets of flasks were included: controls (normal overlay, 36-37 incubation), d flasks (acid overlay, 36-37C incubation), RCT/40 flasks (normal overlay, 40C incubation), and d- RCT/40 flasks (acid overlay, 40C incubation). No precedent for combining the two sets of test conditions in a single flask is known to exist; however, if the acid and high-temperature conditions are truly counterparts of those occurring in infected, inflamed tissue, it would seem that the two would be more likely to occur concurrently than separately in nature.

Holland (8) reported that the neurotropism of polioviruses could be determined by incubating the virus suspension with a homogenate of monkey central nervous system tissue, and determining whether or not the virus was inactivated by this treatment. He postulated that non-neurotropic virus is not inactivated during incubation with central nervous system homogenates because the cells from which these preparations were made lack receptors to which the viruses can adsorb. Homogenates of rhesus monkey spinal cord were first employed, with equivocal results. The test was performed thereafter with a 5% of homogenate of rhesus monkey brain in phosphate buffered salt solution. One volume of virus suspension was mixed with nine volumes of the tissue homogenate and incubated for 2 hr at room temperature prior to assay in tissue culture. At times, the viruses were also incubated with homogenates of cultured monkey kidney cells as a positive control. This was

a deductive test, in the sense that the property we were monitoring (neurotropism) was manifested by the absence of plaques. It therefore could not be used for screening whole suspensions of irradiated virus, but only for assessing the presumptive neurotropism of derived, purified strains.

Swine kidney cell cultures comprised the test system for studying species specificity. Several procedures were employed. The simplest entailed inoculation of the test virus suspension into swine cell cultures, maintained under fluid medium, and daily microscopic observation for cytopathic effects (CPE). Regardless of whether CPE were seen, the cultures could be frozen after periods of incubation and the virus titers of the contents determined. If there was reason to expect virus replication to occur in the absence of CPE, virus which had been photosensitized with neutral red could be inoculated. Progeny virus could then be distinguished because it was not inactivated by a strong pulse of white light. Virus could also be diluted and inoculated into swine cell cultures as in a conventional plaque titration, or a small volume of fluid inoculum was placed at a single site on the top of the agar overlay so that the virus diffused to the swine cells. Adsorption experiments were carried out with both monolayers and suspensions of swine cells (cell suspensions for this purpose were harvested with EDTA from monolayers). Virus could also be adsorbed to PMK monolayers and these cells harvested and assayed as infectious centers on swine cell monolayers. This last approach was suggested by evidence that host range mutants might be detected only after passage in PMK: the infectious centers technique should limit to one the number of replicative cycles through which the virus has passed in PMK before the swine cell challenge.

Several techniques for the neutralisation test were used in attempting to detect and characterize antigenic variants. In one screening procedure,

a disc of blotting paper was dipped in a 1:8 dilution of antiserum and applied to standard overlay (but minus neutral red) in a PMK culture. After overnight incubation, the disc was removed and the agar surface flooded with a suspension of test virus plus neutral red. The flask was held at room temperature for 2 hr with continuous agitation, inverted, and incubated thereafter at 36-37C. In a homologous reaction, an area of approximately 25mm diameter around the disc site was protected, while all cells at the periphery of the culture were killed by the virus. Antigenically deviant virus caused cell-killing within the "protected zone." Virus could also be inoculated directly onto the cell monolayer and, after 2 hours' adsorption, followed with an agar overlay medium containing antiserum. Another agar-diffusion neutralization test was developed under other sponsorship for the purpose of identifying unknown enteroviruses. In this instance, eight diffusion discs, each with a different mixture of antisera, were arrayed on agar overlays in two PMK cultures. The remainder of the method was essentially the same as that of the agar-diffusion screening procedure; however, the pattern of protection or killing at the eight disc sites permitted identification of an unknown enterovirus as one of 40 possible types. The neutralization reaction was also carried out in a test tube. The quantity of virus which had not been neutralized (after one or several intervals of incubation) was determined by the plaque technique in PMK cultures.

RESULTS

Irradiation. In a preliminary test, poliovirus type 1 was exposed to a dosage of 0.5 Mrad and was found to have been inactivated approximately 99%. While this and subsequent inactivations were found to exceed somewhat the levels that would have been predicted by the D value given above, these quantitative differences were determined to be outside the scope of the

present study and were explored. When each of the four virus types was exposed to the dosages described in the preceding section, it was found that in each case 0.5 Mrad resulted in a greater degree of inactivation than did 0.25 Mrad. The limited data obtained did permit precise mathematical formulation of the dose response. Either "directly irradiated" virus or progeny after passage in PMK cells was used in subsequent tests, as appropriate.

Poliovirus type 1 (Po-1) was tested for "virulence" markers. The titer of the unirradiated virus, under standard assay conditions, was 3.8×10^8 plaque-forming units (PFU)/ml. This was reduced to 2.8×10^8 PFU/ml under the conditions of d-marker test, to 2×10^6 PFU/ml in the RCT/40 test, and to $<10^6$ PFU/ml when the d and RCT/40 conditions were imposed concurrently. These results would be scored d^+ , RCT/40⁻, for the reduction in the d test was not significant. The data suggested that the effects of the acid overlay and the elevated incubation temperature might be additive, but the data were insufficient to draw any firm conclusion, and the matter was not investigated further. The "neurotropism" test caused >90% inactivation, which was quite an unexpected result for an attenuated virus. A second trial confirmed this finding.

Antigenically, Po-1 was quite homogeneous. Homologous reactions were obtained consistently in the diffusion-screening test with anti - Po-1 serum, and no cross-reactions occurred when antisera to the other model viruses were used instead. Po-1 was without effect in early and near-term cell cultures.

Irradiation doses of 0.25 and 0.5 Mrad were found to have caused 36% and 99% inactivation, respectively, of Po-1. Our tests revealed no variations from baseline properties in the irradiated Po-1, even after passage in PMK

cells, with respect to the virulence and neurotropism markers.

Though we had shown that Po-1 prior to irradiation did not cause CPE in PFSK cultures, replication of the virus had not really been ruled out. It seemed that this could be done most efficiently by photosensitization of the virus inoculum. Po-1 after dosages of 0, 0.25, and 0.50 Mrad was propagated in PMK cells in the presence of 5 μ g of neutral red per milliliter of maintenance medium (in the dark). The harvested virus suspension was freed of dissolved neutral red by passage through a Sephadex G-25 column, for it had been found that neutral red not incorporated into virus particles could photosensitize subsequent generations of virus. The resulting preparations ranged from ~99% to 99.99% photosensitive when pulsed with light from a fluorescent desk lamp. Each was inoculated into PASK cell cultures (lot 29A). Half of the inoculated PASK cultures also received 10^5 infectious doses of a latent virus which had been obtained from an earlier lot of swine cells and was designated LSCV-1. Only the cultures inoculated with LSCV-1 showed CPE, and none of the harvested tissue culture fluids caused plaque formation in PMK cells. Additional portions of the photosensitized CA-9 suspensions were inoculated into two later lots (34A and 35A) of PASK cells which were thought to contain adventitious swine viruses. CPE, none of which went to completion, were seen only after 9 or more days' incubation. Harvested fluids from lot 35A (but not from lot 34A) cultures inoculated with Po-1 caused plaque formation in PMK cell cultures (<10 plaque-forming units (PFU)/ml). The inocula had not been light-pulsed, so it is not at all certain that the few viruses obtained had been propagated in the PASK cells. More to the point, there was no indication that the individual Po-1 strains' irradiation histories (0, 0.25, or 0.50 Mrad) had any influence upon their ability to multiply in PASK cells, with or without an adventitious swine virus "helper."

Preliminary tests by the agar diffusion neutralization screening technique gave no indication of antigenic variants in the irradiated Po-1 suspensions. Further tests were performed by the tube neutralization, plaque reduction method and by the procedure in which the virus is inoculated onto the cell sheet and the antiserum is incorporated into the overlay medium. No antigenic variants were detected.

Coxsackie virus type A-9 (CA-9), unirradiated, gave a titer of 6.9×10^8 PFU/ml in control cultures of the "virulence" tests. The titer in the d test was 2.3×10^8 PFU/ml, while those for the RCT/40 and combination tests were 3.0×10^8 and 4.3×10^8 PFU/ml, respectively. This indicated that these conditions had only a slight inhibitory effect upon the virus and that the influences of the acidity and high temperature were not additive. No significant loss of titer resulted from incubating the virus with monkey brain suspension, suggesting that the strain was not neurotropic.

Unirradiated CA-9 gave consistently positive reactions with homotypic antiserum and negative reactions with antisera to the other three viruses included in this project. Somewhat later, the virus was also tested with antisera to poliovirus types 2 and 3 and to Coxsackie virus type B-3, which are also used in our laboratory though not in this project, with negative results. Initially, a significant proportion of "variants" was detected in the unirradiated virus by the plaque-reduction neutralization technique, but these were eliminated if the virus suspension was passed through a 50 nm membrane filter (9) before it was mixed with the antiserum. It seemed likely that these "variants," which occurred in proportions as high as 7% in some experiments, were actually aggregates of antigenically typical CA-9. One can envision a continuum of virus aggregates ranging

from simple dimers to those which include enough particles (and possibly some host-cell material) that a portion of the virus is sequestered and inaccessible to the antibody. Dimers, which might conceivably occur in a 50 nm filtrate, are harder to neutralize than single particles principally because, as a matter of statistical probability, each of the component particles must be neutralized before this one presumptive PFU is neutralized. Our neutralization tests provided such a high order of redundancy that this was not likely to be a problem. Large aggregates with sequestered virus presumably diffuse so inefficiently through agar that they are not likely to be a factor in the diffusion-neutralization screening test. Since it was evident that such artifacts were a problem in the plaque reduction test, 50 nm filtration was used in all subsequent experiments.

It was found early that unirradiated CA-9 might cause CPE in PASK cells without demonstrable replication. Adsorption was rarely measurable in PASK but was found to occur in PFSK, though with somewhat less efficiency than in FMK cells.

Inactivations of 80% and 95% at dosages of 0.25 and 0.5 Mrad were observed in the initial irradiation series. The later irradiations of a second set of CA-9 samples at 0.19 and 0.38 Mrad gave corresponding inactivations of approximately 73% and 82%. Clearly, there was not a precise dose-response relationship between irradiation and inactivation as we measured them. We did not carry out a more rigorous set of titrations because it seemed that the degree of precision achieved in these tests was adequate for our purposes. We did perform filtrations at 50 nm on the second set of irradiated samples in an attempt to determine the extent to which aggregation played a part in resistance to γ -rays. The results were not definitive: only 6% of the titer of the unirradiated suspension was lost in 50 nm filtration, and the 0.38 Mrad preparation lost 38% in filtration, but the 0.19

Mrad suspension gained 28%. These data may suggest that a greater proportion of the PFU in the irradiated suspensions consisted of aggregated virus particles, but such a conclusion should be drawn only with extreme caution.

CA-9 after 0.25 and 0.5 Mrad irradiation showed no significant changes in the d, RCT/40, and "neurotropism" markers. The first diffusion-neutralization screening test of irradiated CA-9 (after a single PMK passage) revealed a heterologous reaction between the 0.25 Mrad virus and anti-CA-9 serum. No confirmed variants have been detected in the 0.5 Mrad virus in this or any subsequent experiment. The 0.25 Mrad virus failed to react homotypically with antiserum to any enterovirus in use in our laboratory.

Derivative strains of unirradiated and 0.25 Mrad CA-9 were prepared by subculturing from single plaques formed in PMK cultures. These derivative strains were tested by the diffusion-screening method. The results of the first trial were anomalous. A second experiment revealed no presumptive variants among 11 unirradiated substrains, and two among 11 0.25 Mrad substrains. This may give some indication of the proportion of variants in the virus suspension with which the previous diffusion-screening test was performed.

Thereafter, great emphasis was placed upon the serologic characterization of CA-9 and its irradiated derivatives. Instead of obtaining strains randomly for further study, as was done in the experiment described above, derivative strains were prepared after some selective pressure had been applied. For instance, a plaque reduction, tube neutralization test was performed, and plaques which formed after virus had been incubated with antiserum were subcultured and studied. Some of these were tested to see if this form of selective pressure favored changes in other markers, such as virulence or host range (it did not), but most of the follow-up studies were serologic.

In preliminary, plaque reduction, tube neutralization tests with the second set of CA-9 viruses (0, 0.19, and 0.38 Mrad irradiation), after one PMK passage, we saw no dose-related differences in the "non-neutralizable" fractions. The same preparations were inoculated directly onto PMK monolayers (7×10^3 PFU per flask) and overlaid with agar medium containing 1% CA-9 antiserum. No plaques were formed in any of these cultures. Directly irradiated (0, 0.19, and 0.38 Mrad) CA-9 suspensions were diluted 10^{-3} and inoculated onto PMK cell monolayers and overlaid with 1% antiserum medium. The 0 Mrad inoculum contained 10^5 PFU/ml, and no plaques were formed. The 0.19 Mrad inoculum contained 3×10^4 PFU/ml, and two plaques formed and were subcultured (designated numbers 5 and 6). The 0.38 Mrad inoculum contained 6×10^3 PFU/ml, and a total of 24 plaques formed in six inoculated cultures. Four of these (designated numbers 1-4) were subcultured. Strains 1, 2, 3, 5, and 6 were tested in PMK under 0.7% antiserum agar medium and showed what appeared to be abnormally high (11.5% to 100%) efficiencies of plaquing in the presence of antibody. When the antiserum level in the overlay was decreased further, to 0.3%, even the unirradiated control virus plaqued with 44% efficiency.

A plaque reduction, tube neutralization test was used to compare two of these strains with the directly irradiated suspensions from which they had been derived and with a suspension of unirradiated virus. The results were equivocal. Both the tube neutralization and antiserum in overlay methods were used in a further comparison. The virus preparations included were unirradiated CA-9, the directly irradiated (0.38 Mrad) suspension, and the number 3 substrain derived from it. Antiserum to CA-9 was present at 1% concentration both in the antiserum overlays and in the neutralization tubes. Plaquing efficiencies after tube neutralization were 0.1%, 0.4%, and

0.4%, respectively, for each of the virus preparations listed. The corresponding efficiencies under antiserum overlay were 0%, 0.6%, and 24.0%, respectively. This made it appear that the selective process by which substrain 3 was derived had resulted in a significantly lower level of antibody sensitivity as measured by the one test, but not the other.

The plaque reduction, tube neutralization test was also performed "kinetically." That is, the mixture of virus and antiserum was sampled after various periods of incubation at room temperature, and the samples were assayed individually. A significant liability of this technique is that 2 hr must be allowed for the virus in each sample to adsorb to the PMK cells before the monolayer could be rinsed and overlaid with agar medium. This means that the plaque technique cannot tell us what degree of neutralization had occurred at some point in time in the reaction tube, for the time spent in adsorption is invariably greater than the "reaction" time in the tube. Dilution (100-fold or more in cold buffer) was used in an attempt to arrest the neutralization process at the time of sampling, but one cannot be sure that this was entirely successful.

An extensive series of kinetic neutralization tests was performed. The results were not entirely consistent, but there did seem to be some common features. The concentrations of virus and antiserum had to be carefully selected if any differences in neutralization rate were to be seen at all. In general, irradiated, PMK-passed preparations of CA-9 were neutralized less rapidly than unirradiated CA-9. The strains subcultured from plaques formed under antiserum overlay were generally neutralized more slowly than their parent irradiated virus suspensions. Successive subcultures with selection by neutralizing antibody would apparently yield CA-9 which was neutralized even more slowly. A relatively resistant strain

was derived in one instance from a plaque initiated by a late sample from a kinetic neutralization of an unirradiated CA-9 suspension. With virus at 10^6 PFU/ml and antiserum at 1:300 dilution, "slow" neutralization tended to take the form of a persistent fraction of $> 1\%$ which was not neutralized within 1 hr or even 2 hr. (With virus $< 5 \times 10^4$ PFU/ml and antiserum at 1:400 dilution, the persistent fraction or "tail" was not seen in the kinetic curves, but the degree of neutralisation early in the reaction (~ 10 min) was usually reduced. Ultimately no CA-9 preparation was obtained, regardless of irradiation treatment or intensity of selection, which was entirely refractory to CA-9 antiserum. Differences in neutralization performance were seen with sufficient frequency among the irradiated CA-9 preparations to make us believe that the gamma rays had had some genetic effect, yet the differences were never so great as to have obvious public health significance. That is, it seemed likely that an individual who had acquired immunity to CA-9 through previous exposure would probably be functionally immune to the most atypical of the antigenic variants derived from CA-9 during these studies.

There were also occasional indications of host range variations in irradiated CA-9. When irradiated CA-9, after one PMK passage, was tested in near-term PFSK cultures, CPE were seen only with the virus which had received the 0.25 Mrad dose. This same virus suspension produced CPE in a batch of PFSK cells from a very early fetus; CPE development only partially inhibited by antiserum to CA-9. In later experiments the CPE were generally less complete and much less final than those seen at this stage. We had assumed that virus would have to be adsorbed to the cells in order to initiate a demonstrable infection. While this is undoubtedly true in principle, the degree of replication shown to have occurred in several later experiments might have resulted even if a very small proportion of the cells were capable of adsorbing CA-9. The means at hand did not permit adsorption

to be measured at these extremely low levels.

During the entire course of these studies, no CA-9 plaques have been seen in swine cells. At first there were some toxicity problems, for the overlay medium that we use in our PMK cultures apparently is not compatible with swine cells. Even after this was corrected, CA-9 did not form plaques. Preparations tested included unirradiated, directly irradiated, and irradiated, PMK-passed virus, as well as virus adsorbed to PMK cells and assayed on swine cell monolayers for infectious centers.

Swine kidneys used to prepare tissue cultures are obtained at a local slaughterhouse. A substantial proportion of them have been thought or shown to harbor adventitious viruses. At least one of these, designated LSCV-1 (latent swine cell virus), seems to have some ability to serve as a "helper" for CA-9 replication. It produces a very late CPE of its own (but not plaques), in swine cells is chloroform-sensitive, 100-200 nm in diameter, and (as indicated by acridine orange staining) induces intranuclear, DNA-type inclusions in swine cells. These properties suggest that the agent is a member of the herpes group. It produces neither CPE nor plaques in PMK. Electron micrographs prepared for us neither confirm nor refute this: they show only sufficient detail to make it certain that the agent is a virus. Attempts to cultivate mycoplasma from LSCV-1 have been negative.

As was mentioned above, LSCV-1 was derived from lot 21 A of PASK cells. When inoculated with CA-9, these cells showed an irregular pattern of CPE which was also seen in the uninoculated cell controls. Fluid harvested from the PASK cultures was assayed by the plaque technique in PMK cells. Small plaques were formed when fluids derived from PASK cultures, inoculated with CA-9 which had received 0.19 Mrad or 0.38 Mrad, were tested. PASK cultures which had not been inoculated, or which had received unirradiated

virus, yielded no PFU. The agent(s) which caused these plaques produced CPE when subcultured into PMK with fluid medium, but apparently were lost in this passage.

Two further approaches were taken in trying to define the phenomena. CA-9 was passed alternately between PMK and swine cells in an attempt to adapt it to the latter, and photosensitized suspensions were prepared in an attempt to demonstrate replication. The alternate passage system seemed to result in a fairly stable association of CA-9 with "helper" virus, but performance was erratic due to the intermittent appearance of new adventitious viruses in later lots of PASK. No CA-9 preparation was proved capable of replicating in PASK without a helper virus, and ability to use an adventitious swine agent as a helper was (unfortunately) apparently unrelated to the virus preparation's irradiation history. Continuous swine cell passage would not maintain CA-9 infectivity.

The results of experiments with photosensitized CA-9 were compatible with those just described. In general, the budget in a replication experiment is as follows: one inoculates a known number of virus PFU per tissue culture; these may be either washed out at the end of an adsorption period, inactivated with time during incubation, adsorbed and eclipsed, or replicated. Some of the inoculated virus may simply persist until the culture is harvested. When photosensitized virus is inoculated, this persistent inoculum can be largely ignored; for most of it can be inactivated in the harvested fluid by a light pulse. Ideally, one would prefer that the inoculum be 99.99% photosensitive or more. This was frequently, though not invariably, accomplished. It permitted us to conclude that virus replication had occurred if the quantity of non-photosensitive virus harvested at the end of the incubation period exceeded the quantity of non-photosensitive virus which

had been present in the inoculum, regardless of other "debit entries," such as thermal inactivation. Net increases in non-photosensitive virus have been recorded repeatedly, though in each case the total virus harvested was less than the total inoculated. Such presumptive replication has not occurred in the absence of a "helper" and has not depended upon the irradiation history of the inoculum.

Coxsackie virus type B-2 (CB-2), unirradiated, gave a control titer of 4.5×10^8 PFU/ml in the "virulence" tests. The other titers were 3.5×10^8 PFU/ml in the d test, 1.6×10^8 PFU/ml in the RCT/40 test, and 1.5×10^8 PFU/ml in the combined test. Thus, the influence of elevated temperatures was small, but significant, while the effect of the acid overlay was essentially negligible. One ml of a dilute suspension produced 54 plaques, but only 3 plaques after incubation with monkey brain homogenate. This is interpreted as a positive test for neurotropism.

The agent behaved quite typically in the diffusion-screening test. It gave a homologous reaction with antiserum to CB-2 and a heterologous reaction with antisera to each of the other enteroviruses used in our laboratory. Diffusion-typing tests, performed as part of another project, have always identified this agent as CB-2. Plaque-reduction neutralization tests have shown a significant reaction with antiserum to CA-9. We suspect that this indicates that the commercial CA-9 antiserum contains some antibody to CB-2. Unirradiated CB-2, inoculated into PASK at ca. 500 PFU/culture, destroyed all of the cells within 96 hr and yielded from 4.5×10^8 to 2.0×10^9 PFU/culture when incubated at 36-37C. No cell-killing was seen in inoculated cultures incubated at 40C.

In the original irradiation series, the titers were: unirradiated, 1.1×10^9 PFU/ml; after 0.25 Mrad, 1.8×10^8 PFU/ml; and after 0.5 Mrad,

2.1×10^7 PFU/ml. These are reductions of 84% and 98% respectively, for these irradiation dosages. Titers in the second irradiation series were: control, 2.9×10^8 PFU/ml; 0.19 Mrad, 5.6×10^7 PFU/ml; and 0.38 Mrad, 1.0×10^7 PFU/ml. These are reductions of 81% and 97%, respectively, and are therefore somewhat greater per rad than those in the original series. After filtration at 50 nm, the control titer was reduced 20%, the titer of the 0.19 Mrad suspension, 48%, and titer of the 0.38 Mrad suspension, 0%. As in the case of CA-9, we are not at all sure of what these filtration data prove.

CB-2 after 0.25 or 0.5 Mrad showed no alterations in the "virulence" markers in a limited series of tests. Because it was positive in the "neurotropism" test prior to irradiation, the property was not studied further. Irradiated virus reacted atypically in the diffusion-screening neutralization test. When strains which had been passed in PMK after 0, 0.19, and 0.38 Mrad were inoculated onto PMK monolayers and overlaid with agar medium containing 0.1% antiserum to CB-2, no plaques were formed.

Directly irradiated (0, 0.25, and 0.50 Mrad) CB-2 was inoculated into two lots of PFSK at 100 PFU per culture. Each yielded $> 10^8$ PFU (measured in PMK) per culture except the 0.5 Mrad suspension in one of the two lots of PFSK, which gave $< 5 \times 10^6$. A similar set of virus suspensions, together with a second series which had been passed once in PMK subsequent to irradiation, was inoculated into PASK at 750 PFU per culture. With 37C incubation, all cells were killed within 96 hr, showing typical enterovirus CPE, and the yield from each culture was $\geq 10^9$ PFU. No CPE were seen if incubation was at 40C, so all of these cultures were incubated at 36-37C after the first 132 hr. The PFSK culture which had been inoculated with 0.5 Mrad virus, not passed in PMK since irradiation, was destroyed within 72 hr

after being returned to the 36-37C incubator and yielded 1.0×10^9 PFU. The other cultures which had been at 40C were not killed when the incubation temperature was reduced, nor was virus recovered from them. CB-2 forms plaques in PASK, but with lower efficiency than in PMK. In direct comparisons, the relative efficiency of plaquing in PASK has ranged from 25% down to 0.05%. Irradiation seemed in one instance to have enhanced the plaquing efficiency of CB-2 in PASK, and in another instance to have reduced it. Neither of the PASK lots in which these experiments were run gave any indication of harboring an adventitious virus, so the difference must have been caused by something else.

ECHO virus type 6 (EC-6), unirradiated, presented difficulties because it forms plaques slowly and inefficiently in PMK cultures. Inhibition in the RCT/40 test was $\geq 80\%$, but the acid overlay in the d test generally had little effect. Incubation with monkey brain homogenate resulted in no significant loss of titer. The unirradiated virus gave a homologous reaction with antiserum to EC-6 in the diffusion-screening test and was identified as EC-6 in the diffusion-typing test. The agent failed to induce CPE in PFSK in one experiment, but killed PASK cells in a later experiment.

The titer of a control virus suspension was 1.8×10^8 PFU/ml, while those after irradiation dosages of 0.25 and 0.5 Mrad were 5.1×10^7 and 1.5×10^6 PFU/ml, respectively. These are reductions of approximately 73% and 99%. No definitive change in the "virulence" markers was noted following irradiation, but the bases for comparison were rather inadequate. Both of the irradiated preparations, passed once in PMK, have given heterologous reactions in the diffusion-screening test. No antigenic variants were detected by the plaque reduction, tube neutralization test. EC-6 suspensions, irradiated and passed in PMK, caused CPE in PFSK cells from an early fetus. Not all of the cells were destroyed in these cultures, nor were the CPE typical of those induced by enteroviruses.

DISCUSSION

The virulence and neurotropism "markers" selected for study in this project were originally developed for use with the polioviruses. It is assumed that the properties measured are genetically controlled, that they have some relevance to viral pathogenesis in the whole host organism, and that they are applicable to other enteroviruses. Our experience suggests that one or more of these assumptions may not have been valid. More important, no definitive variation with respect to any of these markers could be attributed to irradiation.

The host range studies in swine cells yielded some very interesting information. Though the propagation of CB-2 in swine cells was not a "first," negative as well as positive results have been reported by others. The possibility that an adventitious swine virus might serve as a "helper" to enable propagation of a human virus in incompetent swine cells has potentially exciting ramifications. The public health significance of such a finding could really be assessed properly only in a whole host organism. Whatever it means, there was no indication that irradiation had anything to do with it.

Only the antigens (and then only those of CA-9 virus) showed a strong likelihood of a radiation-induced variation. The neutralization test may well not have been the one most capable of detecting subtle antigenic variations, but we thought it the test most likely to show the presence or absence of cross-protection among enteroviruses. From this standpoint, the antigenic variations thought to have occurred in CA-9 as a result of irradiation probably lack public health significance. With the exception of some results obtained and reported early in the project (10), all CA-9

virus after irradiation has remained identifiable as CA-9 by the neutralization test. We interpret this to mean that an individual who had acquired active immunity to CA-9 would probably be immune to these "variants" as well.

The survey which we have conducted has not been exhaustive. Enteroviruses are beginning to be detected in market samples of foods, but they most certainly are not the only kinds of viruses with which foods are contaminated. Our selection of "markers" has been restricted to those which could be studied in tissue cultures; how these relate to pathogenesis in the whole host organism is not known. The markers for virulence and neurotropism have led us to question their validity. The ultimate criticism of the host range studies is that we were actually more interested in the opposite situation, in which an enterovirus of animal origin might mutate to ability to replicate in human cells.

Though the program has had its shortcomings, it has resulted in the production of data which did not exist previously. The question which these studies were designed to answer probably can now be given a qualified "no."

SUMMARY

This project was intended to determine whether viruses contaminating foods are likely, with the levels of irradiation which might be used in processing such foods, to mutate in ways that are of public-health significance. The weight of evidence obtained seems to favor a qualified "no."

Dean O. Cliver, Ph. D.

Principal Investigator

LITERATURE CITED

1. Cliver, D. O. Implication of foodborne infectious hepatitis. Pub. Health Rept. 81:159-165, 1966.
2. Cliver, D. O. Food-associated viruses. In Health Lab. Sci. 4:213-221, 1967.
3. Cliver, D. O. Viruses and rickettsia in foods and the possible role of radiation preservation. In Radiation Preservation of Foods. National Academy of Sciences-National Res. Council, Washington, D. C., pp. 269-274, 1965.
4. Sullivan, R., Fassolitis, A. C., and Read, R. B., Jr. Radioresistance of viruses likely to be foodborne. Bact. Proc. 1968:135, 1968.
5. Cliver, D. O., and Herrmann, R. M. Economical tissue culture technics. Health Lab. Sci. 6:5-17, 1969.
6. Carp, R. I., and Koprowski, H. A new test of the reproductive capacity temperature marker of poliovirus: the limited thermal exposure test. Virology 17:71-79, 1962.
7. Hsiung, G. D., and Henderson, J. R. Diagnostic Virology. Yale Univ. Press, New Haven, Conn., 1964.
8. Holland, J. J. Receptor affinities as major determinants of enterovirus tissue tropisms in humans. Virology 15:312-326, 1961.
9. Cliver, D. O. Factors in the membrane filtration of enteroviruses. Appl. Microbiol. 13:417-425, 1965.
10. Cliver, D. O., and Anders, R. J. Apparent "double mutation" induced by gamma rays. Nature 218:187-188, 1968.

virus after irradiation has remained identifiable as CA-9 by the neutralisation test. We interpret this to mean that an individual who had acquired active immunity to CA-9 would probably be immune to these "variants" as well.

The survey which we have conducted has not been exhaustive. Enteroviruses are beginning to be detected in market samples of foods, but they most certainly are not the only kinds of viruses with which foods are contaminated. Our selection of "markers" has been restricted to those which could be studied in tissue cultures; how these relate to pathogenesis in the whole host organism is not known. The markers for virulence and neurotropism have led us to question their validity. The ultimate criticism of the host range studies is that we were actually more interested in the opposite situation, in which an enterovirus of animal origin might mutate to ability to replicate in human cells.

Though the program has had its shortcomings, it has resulted in the production of data which did not exist previously. The question which these studies were designed to answer probably can now be given a qualified "no."

SUMMARY

This project was intended to determine whether viruses contaminating foods are likely, with the levels of irradiation which might be used in processing such foods, to mutate in ways that are of public-health significance. The weight of evidence obtained seems to favor a qualified "no."

Dean O. Cliver, Ph. D.
Principal Investigator