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Uptake of Heterologous Genome by Mammalian Spermatozoa and Its Transfer to Ova through Fertilization

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ABSTRACT Simian virus 40 (SV40) adsorbs on rabbit spermatozoa but does not penetrate the cells, as indicated by the absence of radioactive material seen on autoradiography of spermatozoa exposed to [^{3}H]thymidine-labeled SV40. In contrast, after exposure of spermatozoa to labeled SV40 DNA, radioactive material was found in the postacrosomal area of the spermatozoa. Furthermore, when spermatozoa exposed to SV40 DNA were fused with cells of the CV-1 line of African green monkey kidney cells, infectious SV40 was isolated. After uterine insemination of rabbits with spermatozoa infected with SV40 DNA, both unfertilized and one- and two-celled fertilized ova were obtained. When the fertilized ova were cocultivated with CV-1 cells, infectious virus was recovered. In contrast, CV-1 cells exposed to the unfertilized ova or to zonae pellucidae or polar bodies from the fertilized ova did not show a cytopathic effect. This report provides the first evidence that a heterologous genome can be incorporated into a mammalian spermatozoon and subsequently carried into an ovum during the process of fertilization.

Mammalian ova, both unfertilized (1) and fertilized (2, 3), have been infected with virus. There has, however, been no direct evidence of infection of mammalian spermatozoa by any virus. The purpose of this investigation was to attempt to infect rabbit spermatozoa with a virus and to assess their ability to carry the viral infection into mammalian ova by fertilization.

MATERIALS AND METHODS

Virus and viral DNA

Purified simian virus 40 (SV40) preparations were obtained according to methods described elsewhere (4). Extraction of SV40 DNA and labeling of SV40 DNA with [^{3}H]thymidine followed a technique previously described (4).

Spermatozoa

Rabbit semen was obtained from healthy, mature, New Zealand white bucks by use of an artificial vagina. Ejaculates from two different bucks were usually pooled for use in each experiment. The spermatozoa were washed twice by diluting the semen to a 5.0-ml volume with calcium-free Krebs-Ringer phosphate solution supplemented with penicillin (100 units of potassium penicillin G per ml) and streptomycin (50 μg/ml) (KRPA). The cells were pelleted by 5-min centrifugations at a maximum force of 1000 $\times$ *g*. The supernatant solutions were discarded and the cells resuspended in 1.0 ml of KRPA after the second centrifugation.

Exposure of spermatozoa to SV40 and SV40 DNA

The spermatozoa were diluted to the appropriate concentration in a synthetic medium that has been used for *in vitro* fertilization of rabbit ova (5). The spermatozoa were exposed to unlabeled or [^{3}H]thymidine-labeled SV40 at an input multiplicity of 4×10^6 plaque-forming units per 10^6 spermatozoa in 2 ml of medium. The suspension was incubated for 2 hr with shaking in a Dubnoff metabolic incubator at 37–38°C under 5% CO_2 in air.

The cells were then washed with KRPA and, in some experiments, treated with rabbit anti-SV40 serum at dilutions of 1:50–1:300. The spermatozoa were washed with KRPA and maintained in culture medium for 24 and 48 hr at 37–38°C. In some experiments, spermatozoa were exposed to SV40 DNA or to [^{3}H]thymidine-labeled SV40 DNA at a concentration of 1.0–10.0 μg/10^7 spermatozoa in the presence of 3% (v/v) dimethyl sulfoxide. After incubation of the suspension for 2 hr at 37–38°C, the spermatozoa were washed in KRPA and some of the samples were treated with 100 μg/ml deoxyribonuclease I (DNase), dissolved in synthetic medium, for 0.5 hr at 37–38°C under 5% CO_2 in air.

The spermatozoa were washed again and maintained in culture medium for 24 and 48 hr. The culture medium consisted of the synthetic medium with 20% (v/v) heated (55°C for 20 min) rabbit serum added. The spermatozoa were cultured under 5% CO_2 in air. A decrease of approximately 20% was observed in the motility of the spermatozoa after their exposure to SV40 or SV40 DNA and maintenance in culture for 24 hr. An additional decrease in motility of similar magnitude was observed after 48 hr of culture.

Fusion of spermatozoa with somatic cells

The fusion technique used was essentially that described for fusion between somatic cells (6). [The suggestion that spermatozoa be fused with somatic cells came from Dr. V. Miggiano.] In brief, 10^7 spermatozoa suspended in 0.5 ml of Hank's solution were mixed with 0.5 ml of Hank's solution containing 10^6 of either African green monkey kidney (AGMK) cells in secondary culture or CV-1 cells (7) in the presence of 1000 hemagglutinating units per ml of β-propiolactone-inactivated Sendai virus. The cell mixtures were kept

Abbreviations: KRPA, Krebs-Ringer phosphate buffer with antibiotics, see *Materials and Methods;* AGMK, African green monkey kidney.

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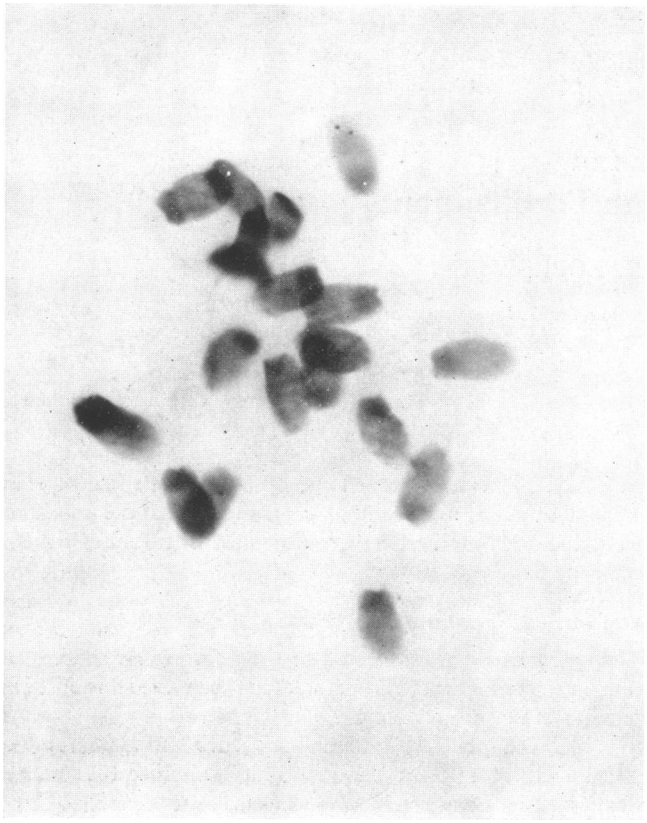

FIG. 1. Autoradiograph of rabbit spermatozoa after exposure to [^{3}H]thymidine-labeled SV40. Harris hematoxylin and Giemsa stain. ×950.

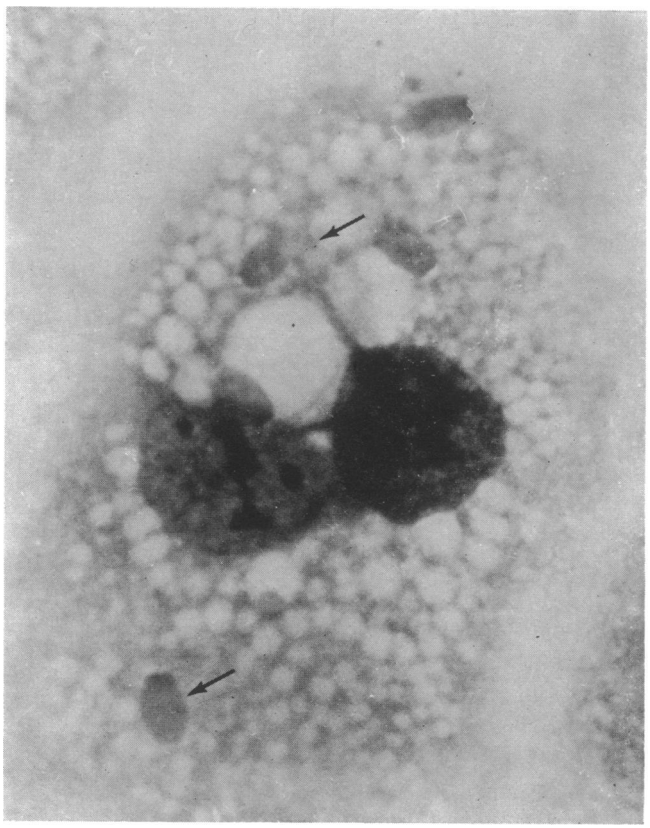

FIG. 2. AGMK binucleated cells showing the cytopathic effect after fusion with spermatozoa infected with SV40 DNA. Note spermatozoan heads (arrows). Harris hematoxylin and Giemsa stain. ×950.

at 0°C for 30 min, 0.5 ml of calf serum was then added to each sample, and the samples were shaken at 37°C for 30 min.

The fused cells were distributed in culture flasks and kept in culture medium with 10% calf serum overnight at 37°C. They were then treated with trypsin to eliminate any unfused spermatozoa that might have remained adsorbed to the surface of the cells. The cells were seeded again in culture flasks and Petri dishes with coverslips and incubated at 37°C. The cultures were observed daily for the appearance of a cytopathic effect characteristic of SV40. For control purposes, spermatozoa not exposed to SV40 or SV40 DNA were also fused with somatic cells so that any cytopathic effect observed could not be attributable to an agent already present in the spermatozoa when the ejaculates were obtained.

Fertilization

New Zealand white rabbit does were superovulated by intramuscular administration of 150 IU of pregnant mare serum gonadotropin ("Gestyl", Organon) followed by 75 IU of human chorionic gonadotropin ("APL", Ayerst) given intravenously approximately 80 hr later (8). At approximately 1.5 hr before the does were treated with HCG, they received an injection into each uterine horn of 3×10^6 spermatozoa (in 0.2 ml volume) that had been exposed to SV40 DNA and resuspended in KRPA.

Approximately 26 hr after uterine insemination, the does were killed by cervical dislocation and ova were recovered by flushing the oviducts. This was approximately 14.5 hr after the commencement of ovulation (9). The fertilized one-celled ova (containing two pronuclei) and the fertilized two-celled ova were collected in either KRPA or 10% serum solution (10). The zonae pellucidae of these and of the unfertilized ova were removed mechanically with a 26-gauge needle. The fertilized ova were also separated (when available) into two blastomeres and polar bodies. The separated cells and their zonae pellucidae were then placed separately on CV-1 cell monolayers in chambers of microtest tissue-culture plates (11). The egg cells were mechanically disrupted, maintained on the CV-1 cell monolayers in Eagle's essential medium with 10% calf serum, and examined daily for cytopathic effect.

Autoradiography

Spermatozoa exposed to [^{3}H]thymidine-labeled SV40 or [^{3}H]thymidine-labeled SV40 DNA were suspended in KRPA, and a smear of the suspension was made on gelatinized glass slides. The slides were dried for about 20 min at room temperature and fixed in ethanol–ether (1:1) for 30 min. Preparations were covered with stripping film and exposed for 5–21 days (12). After development and fixation, the preparations were stained with Giemsa or hematoxylin and eosin, and the percentage of labeled spermatozoa and the mean grain count per cell were determined. The correction for background values of autoradiographs was made according to the method of Stillström (13).

Extraction of labeled SV40 DNA from spermatozoa

Spermatozoa (2.7×10^8) were collected and washed twice with KRPA. They were then exposed to [^{3}H]thymidine-labeled SV40 DNA at a concentration of 1 μg/10^7 cells in the

presence of 3% (v/v) dimethyl sulfoxide for 2 hr at 37–38°C. After a washing with KRPA, the spermatozoan DNA was extracted according to the method of Borenfreund *et al.* (14). The spermatozoa were treated consecutively with ethanol, ether, and 2-mercaptoethanol; they were then centrifuged and their remnants were suspended in 0.01 M NaCl. The radioactivity of the extracted DNA, dissolved in 0.01 M NaCl, was determined and compared with the input radioactivity of SV40 DNA.

RESULTS

Exposure of spermatozoa to SV40

Spermatozoa exposed to SV40 and either left untreated or treated with anti-SV40 serum were fused in several experiments with AGMK or CV-1 cells. Examination of the cultures 24 hr later revealed that 60–90% of the somatic cells contained spermatozoan heads. A generalized cytopathic effect was observed within 72 hr after fusion with the spermatozoa that had not been exposed to antibody. When spermatozoa had been treated with SV40 antibody, however, the cytopathic effect was greatly delayed and occasionally not observed. In one experiment, spermatozoa exposed to SV40 and cultured for 48 hr were sonicated in a sonic oscillator for 9 min at 10 kcycles/sec before fusion with the AGMK cells. The cytopathic effect was again noted after 72 hr.

In several experiments, spermatozoa were exposed to ^{3}H-thymidine-labeled SV40, maintained for 24 or 48 hr in culture, and fixed for autoradiography. The autoradiographs (Fig. 1) showed no radioactivity present in the spermatozoa.

Uptake of SV40 DNA by the spermatozoa

Fusion of spermatozoa exposed to SV40 DNA with AGMK or CV-1 cells resulted in the occurrence of a cytopathic effect, which was first noted on the 12th day after fusion, regardless of whether the spermatozoa were treated with DNase at the time of infection or not. The cytopathic effect was observed first in the cells containing spermatozoa (Fig. 2) and only later did it spread to the adjacent cells. This effect was not observed when uninfected spermatozoa were fused with AGMK or CV-1 cells (Fig. 3).

When a cytopathic effect was observed, the AGMK or CV-1 cells showed the presence of T and VP antigens (15). The cytopathic effect was reproduced within 72 hr when medium from cultures obtained after fusion of infected spermatozoa with CV-1 cells was transferred to uninfected CV-1 cells.

In some experiments, spermatozoa exposed to 1 μg of [^{3}H]thymidine-labeled SV40 DNA per 10^6 cells were cultivated for 24–48 hr and examined by autoradiography. It was found that about 30–35% of the spermatozoa contained radioactive material localized mostly in the post-acrosomal area of the head of the spermatozoon (Fig. 4). The pattern of frequency distribution of grain count per spermatozoon is presented in Fig. 5. The mode of the distribution was 15–18 grains.

In one experiment, after 10^7 spermatozoa were exposed to 1 μg of radioactive SV40 DNA, washed, cultured for 48 hr, and treated with DNase, spermatozoan DNA was extracted

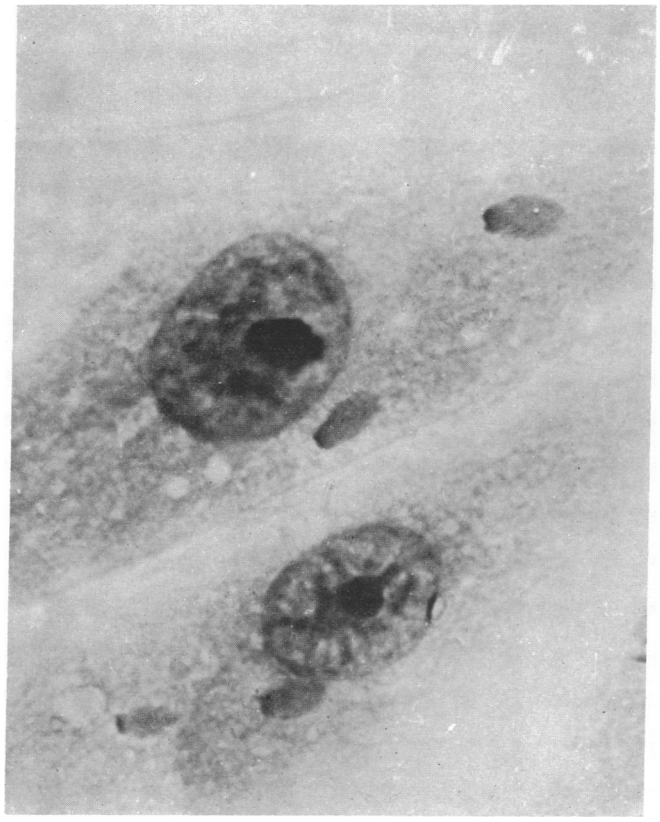

FIG. 3. Uninfected spermatozoa fused with AGMK cells. Note absence of cytopathic effect. Harris hematoxylin and Giemsa stain. ×950.

FIG. 4. Autoradiograph of rabbit spermatozoa after exposure to [^{3}H]thymidine-labeled SV40 DNA. Note the localization of radioactivity in the post-acrosomal area of the spermatozoan heads. Harris hematoxylin and Giemsa stain. ×950.

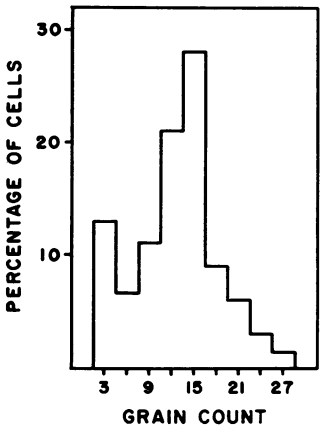

FIG. 5. Histogram of the frequency distribution of the grain count per spermatozoan head after exposure to [^{3}H]thymidine-labeled SV40 DNA.

and analyzed for radioactive content as described above. The results showed that 15.9% of the input radioactivity was recovered from the spermatozoan DNA.

Transfer of SV40

Spermatozoa (6×10^6) were exposed to 6 μg of unlabeled SV40 DNA for 2 hr at 37–38°C and washed once in KRPA. Half of the suspension was injected into one uterine horn of a rabbit, the other half into the contralateral horn. After 26 hr, the recipient animals were sacrificed and the ova were flushed from the oviducts. In the first experiment, 10 of the 28 ova obtained from four rabbits were fertilized (Table 1). The cells were then treated as described in *Materials and Methods* and placed separately on monolayers of CV-1 cells in the chambers of microtest tissue-culture plates.

The cytopathic effect observed in CV-1 cells exposed to fertilized ova was seen in 9 out of 23 chambers in two experiments (Table 1). The cytopathic effect was not seen in CV-1 cells in contact with unfertilized ova or with zonae pellucidae, or in polar bodies obtained from fertilized ova. The number of infected blastomeres from fertilized ova (9 out of 23) was compared by the χ^2 test with the number of infected vitelluses of unfertilized ova (0 out of 29) and the difference was statistically significant ($\chi^2 = 11.3$, $P < 0.001$).

The presence of SV40 in the cultures was confirmed by the presence of T antigen in the CV-1 cells and by the reproduction of the cytopathic effect in uninfected CV-1 cells exposed to medium transferred from cultures showing a cytopathic effect after direct contact with fertilized ova.

DISCUSSION

Although a cytopathic effect was observed in cultures of AGMK or CV-1 cells fused with spermatozoa exposed to SV40, and although SV40 was isolated from these cultures, it is doubtful that the recovered virus was actually localized within the spermatozoa. The fact that when spermatozoa were treated with SV40 antibody after exposure to SV40, the appearance of the cytopathic effect was either greatly delayed or did not occur at all, seems to indicate that the virus particles recovered after fusion with somatic cells were adsorbed to the surface of the spermatozoa without further penetration. This hypothesis is substantiated by the absence of silver grains over the cells after exposure of spermatozoa to labeled virus. If the spermatozoon does not permit penetration of the virus into its interior, then the behavior of this cell differs from that of somatic cells of the same species. The virus can be taken up by these and other nonpermissive cells, and the parental virus can be recovered from the nuclei of the infected cells (4, 16). One- or two-celled ova obtained from a nonpermissive host, such as a mouse, can, in contrast to rabbit spermatozoa, be infected with SV40 (results to be published).

On the other hand, rabbit spermatozoa that were resistant to SV40 uptake became "infected" with SV40 DNA, which could be recovered from the spermatozoa after fusion with SV40-susceptible somatic cells. In contrast to the results obtained after fusion of somatic cells with spermatozoa exposed to SV40, the cytopathic effect in those fused with spermatozoa infected with SV40 DNA was first observed at a much later time after fusion. In addition, whereas a generalized cytopathic effect, probably caused by a massive exposure to virus particles adhering to the surface of the spermatozoa, was noted in somatic cells after fusion with spermatozoa exposed to SV40, the cytopathic effect observed in somatic cells fused with spermatozoa exposed to SV40 DNA was first limited to cells that contained spermatozoan heads, and only thereafter did it spread to the adjacent areas. The fact that the spermatozoa were treated with DNase after exposure to SV40 DNA makes it highly probable that the cytopathic effect was caused by DNA isolated from the cell rather than by DNA adsorbed to the surface of the spermatozoon. Intracellular localization of the SV40 DNA was further confirmed by the results of autoradiography, which showed accumulation of grains over the post-acrosomal area of the spermatozoan heads. Localization of the label in a selective region of the nucleus was similar to that observed by Darzynkiewicz *et al.* (17), who found that [^{3}H]actinomycin D, after penetration of the membrane of the testicular spermatozoon, is localized in the same region.

Perhaps the best evidence for the presence of biologically active SV40 DNA in the spermatozoa was provided by its recovery from the fertilized ova. This transfer of SV40 DNA during fertilization is the first recorded instance of such an event taking place in the mammalian organism. Lack of precedent makes it difficult to interpret these results critically. It is possible, however, that the proportion of spermatozoa carrying SV40 genome was far greater than the 30% figure obtained in autoradiography, because when somatic cells are

TABLE 1. *Isolation of SV40 in CV-1 cells exposed to rabbit ova after fertilization with spermatozoa infected with SV40 DNA*

Expt. no.	Material	Cytopathic effect
1	Unfertilized ova	0/18
	Zona pellucida*	0/5
	Polar bodies	0/5
	Fertilized ova†	4/10‡
2	Unfertilized ova	0/11
	Zona pellucida*	0/3
	Polar bodies	0/6
	Fertilized ova†	5/13‡

* Separated from either fertilized or unfertilized ova.
† Two-celled ova or one-celled ova containing two pronuclei.
‡ Separated cells.

exposed to labeled SV40 or SV40 DNA, autoradiographic determination of the proportion of infected cells is a less sensitive technique than demonstration by immunofluorescence of SV40-induced antigen in the cell.

Spermatozoa are flagellate, haploid cells, containing an extremely electron-dense nuclear chromatin in which the DNA·protein complex exists in a state unlike that of any other mammalian cell (18). Since evidence for the existence of DNA in a chemically dynamic state is, at present, sketchy (19), it is impossible even to speculate on the degree of incorporation or integration of heterologous DNA into spermatozoa. The results presented in this paper, however, indicate the advisability of further exploration of the possibility of transfer of heterologous DNA through the fertilization process into mammalian ova and observation of the fate of the embryo developing from treated ova.

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