Electron-Microscopic Examination of Human Milk Particularly from Women Having Family Record of Breast Cancer.* (18168)

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Mouse mammary carcinoma is caused by a filterable, thermolabile agent, which is transmitted from one generation to another through the milk of nursing females(1-3). Newly born female mice which ingest the pathogenic agent with the milk of their mothers remain in good health until they reach middle age; at that time the agent becomes activated(4), causing the development of mammary carcinomas. Young, nursing female mice which carry the tumor agent, and are earmarked to develop mammary carcinomas later in life, show therefore no symptoms of tumors, and appear to be in perfect health at the time they nurse their offspring; yet they discharge the tumor agent in their milk. Only their family record, if available, and particularly if it extends over the period of several preceding generations, may reveal a history of mammary carcinomas in some of the female ancestors on the mothers' side, indicating thereby that such nursing mice may themselves be carriers of the mammary carcinoma agent.

Recently, small spherical particles, having a diameter varying from 20 to 200 mμ, have been visualized with the aid of an electron-microscope in samples of milk obtained from nursing mice known to carry the mammary carcinoma agent(5,6). Similar spherical particles have also been found in tumor cells(7), or in tumor cell extracts(6,8) prepared from either spontaneous, or transplanted mouse mammary carcinomas. Electron microscopic examination of milk(5,6) from mice of families free from spontaneous mammary carcinomas, as well as that of extracts(6,8) prepared from normal mammary glands of such mice, showed only small quantities of spherical particles in some of the samples(6,8), or none at all(5). The true nature of the spherical particles revealed with the electron microscope in the milk of nursing mice known to carry the mammary tumor agent remains yet to be determined, although some investigators are already inclined to feel that these particles actually represent the mouse mammary carcinoma virus(5). Since the possibility is at hand that human breast cancer may also be caused by factors similar to those responsible for the development of mammary carcinoma in mice(4,9), a series of preliminary experiments, reported in this study, has been carried out, with the purpose of determining whether spherical particles, similar to those found in mouse milk, would, perhaps, also be found, with the aid of an electron microscope, in samples of human milk, particularly in those obtained from women having a family record of breast cancer.

Materials. Human Milk Samples. Young, healthy women who had delivered 3 to 7 days

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previously, and were nursing at the time, were interviewed as to the occurrence of malignant tumors in members of their respective families. Two groups of women were selected as donors, as follows:

Group A. Ten milk samples were collected from nursing, healthy women, whose sisters, mothers, or grandmothers, had breast cancer.

Group B. Thirty-two control samples were collected from nursing, healthy women, with a family record apparently free from malignant tumors for 2 preceding generations.

Preparation of Milk Samples for Electron Microscopy. The milk samples were in most instances processed immediately; in 2 instances only, the samples, having been obtained over the weekend, were stored in a refrigerator at 7°C for a few days prior to processing. Several methods have been successively tried in the preparation of the milk samples for electron microscopy. At first, encouraged by the apparently satisfactory results obtained by Graff(5) and his colleagues in processing mouse milk samples for electron microscopic examination, we used a similar method. This procedure was as follows:

Method I. Ten ml of milk were centrifuged at 866 g (2,000 rpm) for 15 minutes to remove most of the fat. To the extracted milk 5 ml of phosphate buffer pH 7.5 was added containing 10 mg of chymotrypsin. This was mixed, incubated for 2 hours at 37°C and allowed to stand at 4°C overnight. The supernatant fluid was decanted from any precipitated protein and extracted with 15 ml of hexane to remove any remaining fat. One and a half ml of this solution was then centrifuged at 50,000 g (32,000 rpm) for 15 minutes. The resulting supernatant was drawn off by means of a curved needle and discarded. The precipitate was resuspended in 0.75 ml of distilled water, using a high speed stirring motor; the resulting suspension was again centrifuged at 50,000 g (32,000 rpm) for 15 minutes. The supernatant was discarded and the sediment was resuspended in 0.75 ml of distilled water, and centrifuged at 10,000 g (15,000 rpm) for 15 minutes, followed by 50,000 g (32,000 rpm) for 2 minutes. The supernatant was now removed, placed in a clean centrifuge tube and diluted to 0.75 ml with distilled water. A holder containing an electro-mesh screen was then carefully introduced into the tube and a final centrifugation at 50,000 g (32,000 rpm) for 10 minutes deposited the material, to be examined, directly on the film supported by the electro-mesh screen. The screens were now removed, air dried, and after a preliminary direct electron microscopic examination, they were shadowed in a vacuum evaporator, at an angle of 10 to 15° at 10 cm distance, using 25 mg of chromium. In view of the fact that the results obtained with this method of segregation were not entirely satisfactory, much of the undifferentiated material remaining on the electron microscopic screen, another method was tried, apparently with more success. This second method was as follows:

Method II. Ten ml of milk were mixed with 30 ml of hexane in a separatory funnel at room temperature and allowed to settle for 15 minutes. After this period of time the bottom part of the mixture was removed from the funnel and centrifuged at 866 g (2,000 rpm) for 15 minutes. Five ml of the fat-free milk was now homogenized in a high speed tissue homogenizer with 35 ml of pure monochlorobenzene (specific gravity 1.12) for 5 minutes. The resulting emulsion was then centrifuged at 866 g (2,000 rpm) for 15 minutes. Three portions were then formed in the centrifuge tube, an aqueous supernatant layer, a middle layer of monochlorobenzene and a precipitate at the bottom. The middle layer was removed by means of a curved needle, by aspiration, and placed into a clean centrifuge tube. This was centrifuged at 10,000 g (15,000 rpm) for 15 minutes. The supernatant was removed, and monochlorobenze added to make a total volume of 0.75 ml. This was again centrifuged at 17,500 g (20,000 rpm) for 10 minutes, the supernatant withdrawn, placed in a clean centrifuge tube containing the holder and electro-mesh screen, and once more monochlorobenzene was added to make a total volume of 0.75 ml. A final centrifugation was made at 50,000 g (32,000 rpm) for 3 minutes. The screen was now re-

TABLE I. Results of Electron Microscopic Examination of Milk Samples Collected from Healthy, Nursing Women, Having a Family History of Breast Cancer. (Group A).

Donor's record No.	Donor's family history	Results of electron microscopic examination of the milk samples*
1-A	Mother's 2 sisters had breast cancer	Particles present in fairly large numbers in clusters (+).†
2-A	Mother had breast cancer	Particles present in fairly large numbers in pairs and clusters (+).
3-A	Sister had breast cancer	Particles present in large numbers in clusters (++).
4-A	Mother and sister had breast cancer	Particles present in fairly large numbers (+).
5-A	Sister had breast cancer	Particles present in large numbers in pairs and clusters (++).
6-A	Mother had breast cancer	Particles present in large numbers in clusters (++).
7-A	Grandmother and 2 maternal aunts had breast cancer	Particles present in large numbers in pairs and clusters (++).
8-A	Mother had breast cancer	Particles present in large numbers in pairs and clusters (++).
9-A	Mother had breast cancer	Particles present in fairly large numbers (+).
10-A	Mother had breast cancer	Particles present in fairly large numbers (+).

* Five of these samples were segregated by method I, and 5 by method II.
† Explanation of symbols: + particles present in fairly large numbers; ++ particles present in large numbers.

moved, air dried, and, after a preliminary direct electron microscopic examination, was shadowed in vacuum, at an angle of 10 to 15° from 10 cm distance with 25 mg of chromium. This second method of segregation proved to be more satisfactory than the first one, the background being clearer and containing less of undifferentiated debris. Of the 10 milk samples of group A, 5 were segregated by method I, and 5 by method II. Of the 32 control milk samples of group B, 9 were segregated by method I, and 23 by method II. Altogether, a total of 396 electron microphotographs was taken from all of the 42 human milk samples examined.

Experimental. Results of electron microscopic examination of human milk samples. Electron microscopic examination of 10 human milk samples collected from women having a family record of breast cancer (Group A), revealed in all 10 samples (Table I) the presence of small spherical particles (Fig. 1-5). These particles had a high density† to the electron beam; they appeared to have a regularly round shape, and a smooth surface; their diameter varied from 20 to 200 mμ. These particles were found in at least some of the electron microscopic fields in pairs (Fig. 3), or clusters (Fig. 2, 4, 5). In 5 of the 10 samples examined, the particles were quite numerous. Essentially, they appeared similar to those found with the aid of an electron microscope in mouse milk(5,6) known to contain the mouse mammary carcinoma agent (Fig. 6).

The electron microscopic examination of 32 human milk samples that had been collected from women having a family record apparently free from malignant tumors for 2 preceding generations (Group B), revealed (Table II) in 11 of the 32 samples examined, the presence of multiple spherical particles, some of them in pairs, or clusters; in 5 of these 11 samples, the particles were in large numbers. Of the remaining 21 control samples of this group, the electron microscopic examination revealed in 17 samples the presence of only very few, mostly isolated, spherical particles (Fig. 7), occurring singly in some of the electron microscopic fields among masses of undifferentiated debris. In size and form, these particles were not different from those previously described,

† The density of the particles to the electron beam was determined by a direct electron microscopic examination of the film, prior to shadowing.

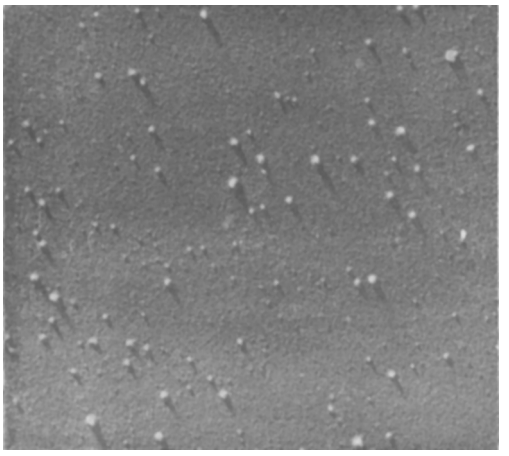

FIG. 1.
Electron-micrograph of a milk sample from a healthy, nursing woman, whose sister had cancer of breast (Rec. 5-A, Table I). Numerous spherical particles present frequently arranged in pairs or clusters. Chromium shadowed. ×10,000.

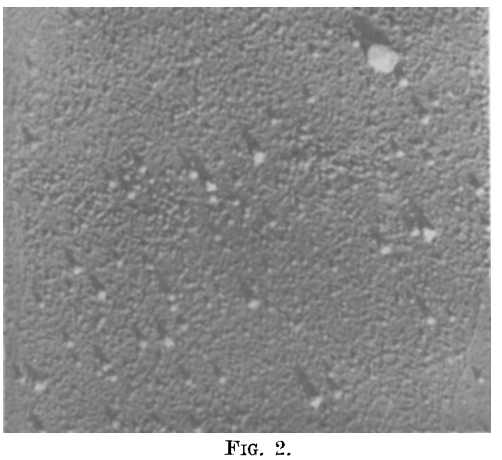

FIG. 2.
Another electron-micrograph of same milk sample as that in Fig. 1 (collected from a woman whose sister had breast cancer). Multiple spherical particles in clusters. Chromium shadowed. ×10,000.

except that they occurred only here and there, in most instances singly, and that at least some of them appeared to have a shallow notch on the top. Finally, 4 samples of milk of this control group appeared to be free from spherical particles, but contained some undifferentiated debris.

Discussion. Experiments reported in this study suggest that milk samples collected from young, healthy, nursing women, whose sisters, mothers or grandmothers had breast cancer, but frequently also from those having a family record apparently free from tumors for 2 preceding generations, may contain spherical particles which can be visualized under an electron microscope. These particles have a diameter varying from 20 to 200 mμ. They have a smooth surface, a high density† to the electron beam, and occur, in some instances

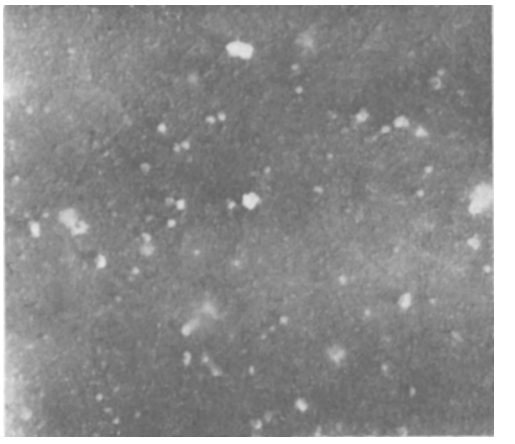

FIG. 3.
Electron-micrograph of a milk sample from a healthy, nursing woman, whose grandmother and 2 maternal aunts had breast cancer (Rec. 7-A, Table I). Spherical particles in pairs and clusters. Chromium shadowed. ×10,000.

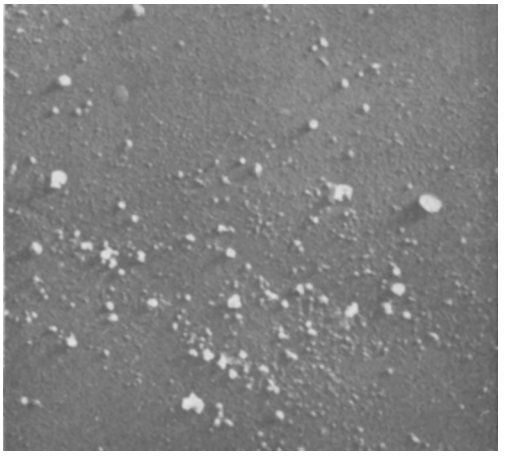

FIG. 4.
Electron-micrograph of a milk sample from a healthy, nursing woman, whose mother had breast cancer (Rec. 8-A, Table I). Numerous spherical particles in clusters. Chromium shadowed. ×10,000.

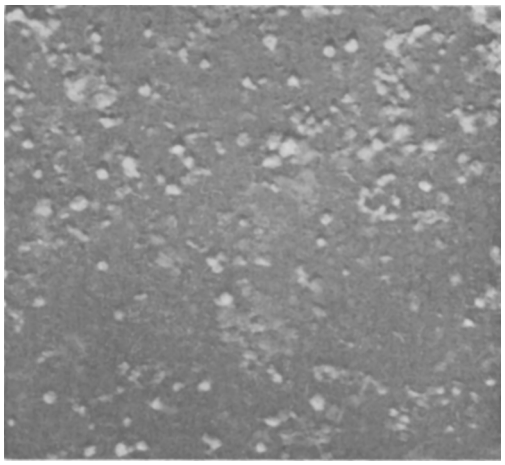

FIG. 5.
Electron-micrograph of a milk sample from a healthy, nursing woman, whose sister had breast cancer (Rec. 3-A, Table 1). Numerous spherical particles in clusters. Chromium shadowed. ×10,000.

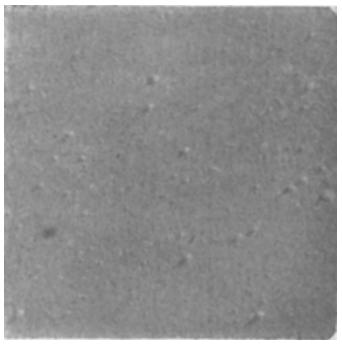

FIG. 6.
Electron-micrograph of a *mouse* milk sample containing the mammary carcinoma agent. This sample was obtained in the following manner: Five litters consisting of a total of 24 suckling infants, 4 to 6 days old, born to mice of the C3H line known to carry the mammary carcinoma agent, were sacrificed; the cheese-like contents were removed aseptically from the stomachs of these infants, and mixed in a mortar with 10 ml of physiological saline solution; the resulting milk-like suspension was then treated exactly like a human milk sample by our second method of segregation. Chromium shadowed. ×11,200. Numerous spherical particles, having a diameter varying from 20 to 200 mμ, very similar to those found in human milk (compare with Fig. 1 and 2).

at least, in pairs, or clusters. In their morphology, size, and group arrangement, these particles appear to be similar to those observed in milk collected from mice known to carry the mammary carcinoma agent(5,6).

The nature of these particles remains obscure. Theoretically, it would be possible to assume that they are (a) normal components of human milk, or that they (b) represent some accidental virus infection unrelated to cancer, or that they (c) actually represent a human cancer agent, essentially similar to the mouse mammary carcinoma virus. It is also possible to assume that (d) some of the particles, such as those present in large numbers, in pairs and clusters, may represent the tumor agent, while other particles, similar in certain respects, but slightly different in others (such as those having a notched top, isolated appearance, etc.) may be either normal components of human milk, similar to those described in normal mouse cells(7), or may have some other origin; small spherical particles have been found with the aid of the electron microscope in various biological samples, recently also in some samples of cow milk(10,11) as well as in samples of mouse milk apparently free from the mammary carcinoma agent (6,11).

Should all these particles be normal com-

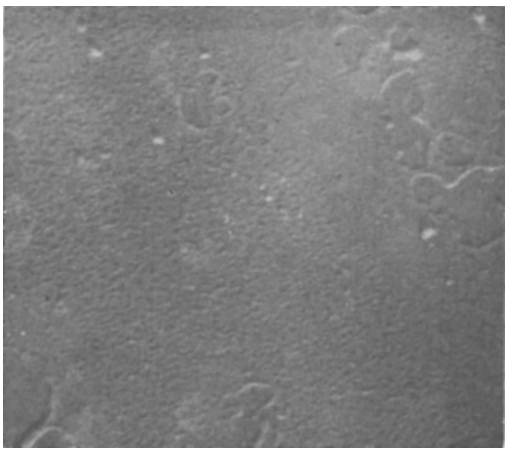

FIG. 7.
Electron-micrograph of a control milk sample, collected from a healthy, nursing woman, who had a family record apparently free from cancer. Only occasional, few particles present in some of the electron-microscopic fields. Chromium shadowed. ×10,000.

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TABLE II. Results of Electron Microscopic Examination of Milk Samples Collected from Healthy, Nursing Women Having a Family History Presumably Free from Cancer. (Group B).

No. of milk samples examined (each from a different donor)	32
Results of electron microscopic examination	
No. of samples* with numerous particles in pairs and clusters (++)	5
No. of samples† with less numerous particles, in pairs or clusters (+)	6
No. of samples‡ with only occasional single particles	17
No. of samples§ with apparently no particles present	4

* 4 of these samples segregated by method I, and 1 by method II.

† One of these samples segregated by method I, and 5 by method II.

‡ 4 of these samples segregated by method I, and 13 by method II.

§ All 4 samples segregated by method II.

ponents of human milk, one would rather expect them to be present in fairly equal numbers, and group formations, in all of the samples examined. This was not the case, however. These particles were present in fairly large numbers, and quite often in characteristic pairs, or cluster formations, in all of the 10 milk samples collected from women having a family record of breast cancer. Of the 32 control samples, however, collected from women having a presumably negative family history for cancer, only 11 contained the characteristic groups of spherical particles in either fairly large, or large numbers; the remaining 21 control samples contained in most instances only single, isolated particles in some of the electron microscopic fields (17 samples), or apparently none at all (4 samples). It also remains open to question whether all of the donors of these 32 control samples were actually descendants of families free from cancer; tumors, including cancer of the breast, might well have developed in some of the ancestors of these women-donors, even though this information might not have been available to the interviewing physicians.

The presence of spherical particles in human milk samples is interesting in view of similar findings recorded in mice. Spherical particles, similar to those reported in this study, were found in samples of milk collected from mice known to carry the mammary carcinoma agent(5,6). When sediment containing such particles was injected into susceptible mice, mammary carcinomas developed in these animals as a result of inoculation(5,6). When normal mouse tissue(6,8), or normal mouse milk(5,6,11) from mice of low-tumor lines, were examined with the electron microscope, spherical particles were also found, but less frequently(5,6,8,11).

Similar spherical particles were also observed with the electron microscope in human cancer(12,13), but less frequently in normal, healthy, human tissues(13).

The wide range of the diameter size of the particles observed in human milk, as well as of those found in mouse milk and mouse tumors (20 to 200 mμ) may, at first, appear to speak against the assumption that these particles represent a tumor agent. And yet it should be kept in mind that electron microscopic examination of several viruses, such as that of mumps(14), or psittacosis(15), also revealed wide variations in their respective sizes.

Interesting as these considerations may be, it should be emphasized, nevertheless, that no definite conclusions can be reached at the present time concerning the nature of the particles observed in human milk. They may, or may not be associated with the hypothetical cancer agent. The only conclusion possible at the present time is that the electron microscopic examination of 10 samples of human milk collected from women having a family history of breast cancer revealed the presence of small spherical particles more frequently than that of 32 samples obtained from women with a family record apparently free from cancer.

Summary. 1. Ten samples of human milk collected from young, healthy, nursing women, whose sisters, mothers, or grandmothers had

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breast cancer, were examined with the aid of an electron microscope. Spherical particles, of a smooth surface and a high density† to the electron beam, varying in diameter from 20 to 200 mμ, in some instances grouped in pairs, or clusters, were found in all samples; they were particularly numerous, however, in 5 of the 10 samples examined. These spherical particles appeared to be similar to those previously observed in mouse milk known to contain the mouse mammary carcinoma agent.

2. Thirty-two control human milk samples, collected from young, healthy, nursing women having a family record apparently free from any malignant tumors for 2 preceding generations, were also examined with the aid of an electron microscope. Eleven of them were found to contain spherical particles essentially similar to those described above. Of the remaining 21 control milk samples, 17 were found to contain only occasional, isolated, single particles in some of the electron microscopic fields; the other 4 samples appeared to be free from spherical particles, but contained some unidentified debris.

3. No definite conclusions can be reached at this time as to the nature of the spherical particles revealed with the aid of the electron microscope in human milk.

Twenty-nine human milk samples were processed, and examined with an RCA electron microscope type EMB at the Research Laboratories of the Interchemical Corporation, New York; Mr. M. Parkinson, Mr. J. J. Kelsch, Mr. R. Bainbridge, and Miss Joan Bardet rendered valuable technical assistance in this part of our work. Thirteen human milk samples and several mouse milk samples were examined with an RCA electron microscope type EMU-2B, at the Research Laboratories of the Veterans Administration Hospital, Bronx, New York.

Most of the human milk samples were obtained from the New York Lying-In Hospital. Several samples were obtained through the courtesy of Dr. Milton J. Goodfriend, New York.

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Relative Susceptibility of Various Stocks of Mice to Experimental Disseminated Encephalomyelitis.* (18169)

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The present paper extends recent studies on experimental disseminated encephalomyelitis induced in white mice by means of injection of homologous brain plus Freund-type adjuvant(1) and offers further results of tests on the variation of susceptibility of certain stocks of mice. Certain aspects of the experimental affection will also be shown. A wide difference has been found(1) in the susceptibility of the W-Swiss(2) and the Rockefeller Institute(3) strains of mice, the former being susceptible, the latter resistant. Thus in an experiment in which the same method and materials were employed, 19 of 20 injected Swiss mice, while only 1 of 15 Rockefeller Institute mice developed encephalomyelitis(1).

The tests now to be described included a study of additional stocks of mice: (a) W-Swiss mice, H-line, of the same strain as was hitherto employed and found to be reactive to inoculation of homologous brain with development of encephalomyelitis. (b) Another stock was added, W-Swiss B-line, obtained from a source outside of the laboratories but

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† Public Health Research Fellow of the National Institute of Mental Health.

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