([a]) The Gregor Mendel Institute of Medical Genetics and Twin Studies, Rome, Italy
(Director: Prof. Luigi Gedda)

([b]) Institute of General Pathology, University of Rome, Italy
(Director: Prof. D. Merlini)

VIRAL ETIOLOGY OF LEUKEMIAS

Electron-Microscope Observation of Virus-Like Particles

([a]) M. TORRIOLI, G. TORRIOLI-RIGGIO, L. BRACONI-ROMEI, E. TORRIOLI
and
([b]) D. MERLINI, M. RUSSO, A. CONFORTI

SUMMARY

The injection of human leukemic blood in chicken embryos before incubation determines a characteristic reticuloendotheliosis, which may be transmitted from embryo to embryo and even to the adult fowl. The viral agent responsible for this reticuloendotheliosis is filtrable and inactivated by heat. Intradermal injection of affected-embryo homogenates in leukemic patients determines a local allergic reaction. Electron microscopy has shown the existence of virus-like particles in the cells of inoculated embryos.

INTRODUCTION

The present paper represents the continuation of a series of researches started over thirty years ago (Torrioli and Riggio 1941): there fore, previous steps and findings shall be briefly reviewed.

The clinical experience had led the senior author to believe that the leukemic disease may be caused by a pathologic agent behaving as a virus. Experiments by other authors having thus far given negative results, he thought that this could be ascribed to the medium. Leukemia affecting bone marrow stem cells, the culture medium should also consist of very undifferentiated cells: therefore, fertile chicken eggs were chosen, inoculated *before incubation*, the allantochorion probably already being too differentiated.

The technique used was very simple: 5-10 ml of citrated blood was drawn from a leukemic patient (either untreated or in relapse) and centrifuged at 400 rpm for 5′. The buffy coat was aspired and diluted in distilled water, until a white-cell concentration of about 10 000/ml was reached. The egg shell was then pierced below the air chamber and 0.1 ml of the white-cell suspension was inoculated. Serial egg-to-egg transmission was carried on employing a homogenate of inoculated embryos 7-8 days old, with uniform results even after 6-7 passages.

Rec. 18. X. 1973

Acta Genet. Med. Gemellol. (1973), 22: 99-107

Long-term observation with different leukemic strains gave the following indications (Torrioli and Torrioli-Riggio 1951*b* and 1953, Torrioli et al. 1953*a*, Torrioli et al. 1953*b*):

(1) A very high embryo mortality and morbidity, with a maximum between the 6th and the 14th day of incubation;

(2) Histopathological findings of reticuloendotheliosis, with a remarkable development of the extraembryonic vascular system;

(3) Loss of the normal erythropoietic activity of the yolk sac;

(4) Formation of extravascular muffs composed by hemohistioblasts, erythroblasts, and granuloblasts;

(5) Mesenchymal alteration of the allantochorion with histioid perivascular muffs.

The aim of the research was to define the characteristics of the pathogenic agent responsible for the embryonic reticuloendotheliosis. This was shown (*a*) to be inactivated by heat at 64° C for 8-10 hours (Cardinali 1947); (*b*) not to be influenced by filtration through S-type Seitz (Torrioli et al. 1951, Torrioli et al. 1953*b*); and (*c*) to be able to induce a disease in the adult fowl as well, when the material employed was an inoculated embryo homogenate at the 6th-7th egg-to-egg transmission, i.e., when the virus had presumably adapted itself to the new environment (Torrioli and Sensi 1949). The disease developed by the chicken was very similar to that observed in the embryo, although the marrow and splenic changes were less evident and the disease less severe, death having seldom occurred.

In 1951, the dead-embryo homogenates were used for an intradermal injection in leukemic patients, using as a control injection the homogenate of embryos inoculated with normal blood. A local allergic reaction was observed when the pathologic material was injected, whereas no reaction was noted to the simultaneous injection with control material (Torrioli-Riggio and Cardinali 1951).

Researches were resumed in recent years, thanks to the support of Snia Viscosa and Generale Immobiliare and to the hospitality of the Mendel Institute in Rome, and led, in 1972, to a collaboration with the Institute of General Pathology of the University of Rome, where electron microscopy was carried out.

MATERIAL AND METHODS

Virus-and mycoplasm-free chicken eggs from two different breedings, one from Halifax and the other from Cuxhaven, were used. Previous findings were all corroborated: death and morbidity rate, heat inactivation, filtration (this time a millipore 400 was used), and intradermal reaction in leukemic patients (Torrioli et al. 1970). The technique for intradermal injection was modified: whereas in 1950 the homogenate was used as such, in 1970 it was previously inactivated by heat, centrifuged at 3 000 rpm for 30′, acidified to pH 1, and subsequently brought again to pH 7 under a germicide lamp for 24 hours.

The chicken eggs were divided into the following five groups:

(1) Normal chicken embryos;
(2) Embryos inoculated with normal human blood;
(3) Embryos of this second group after several egg-to-egg transmissions;
(4) Embryos inoculated with human leukemic blood;
(5) Embryos of this fourth group after several egg-to-egg transmissions.

The above material was then submitted to blind-test electron microscopy, carried out as follows. Specimens were fixed in a glutaraldehyde solution at 2% for 3-12 hours, postfixed with OsO_4, 1.33%, rapidly dehydrated and embedded in Epon 812; sections cut with diamond knives, stained with uranyl acetate and PbOH, were observed with a Philips EM-300. Free cells from cultures were treated with the same procedure by centrifugation.

RESULTS

In the egg membranes of the infected embryos striking changes were noticed in the perivascular cells. These alterations consisted of irregular clumping of nuclear chromatin, irregular density of cell sap, swelling, fragmentation and degranulation of the R.E.R., breaking off of polysomes, and « intermediate » conformation of mitochondria (cf. Hackenbrock et al. 1971).

Sometimes, as shown in Figs. 1-3, virus-like particles (VLPs), with characteristic morphologic features and localization, could be noted: round in shape, the average diameter ranging from 800 to 900 Å units, with a dense core structure surrounded by a lighter zone delimited by a double membrane; rather scarce, sometimes lying in the cell sap (Figs. 1B and 3A), more often in the R.E.R. cisternae (Figs. 1A, 2A, and 2B), and rarely in the extracellular space near the plasma membrane (Fig. 3B).

Other pathological features were noted, not referable to any cellular alteration, i.e., (*a*) groups of round particles, averaging 500 Å in diameter, composed by a membrane envelope containing a material of variable density (Fig. 4A); and (*b*) large membrane whorls embedded in a dense matrix and tending to form round envelopes at the outer face (Fig. 4B).

DISCUSSION AND CONCLUSIONS

Attempts to demonstrate the presence of VLPs in human leukemic blood and their serial transmission to animal cells have already been made by other authors.

As a matter of fact, VLPs have already been described in leukemic blood sera and isolated and cultured cells (Porter et al. 1964, Anderson 1965, Dmochowski et al. 1967, Levine et al. 1967, Smith et al. 1967, Newell et al. 1968, Seman and Seman 1968). Negative results have been obtained in serial transmission experiments by Smith et al. (1967), while successful results have been obtained by Magrassi et al. (1969), though raising doubts on their origin and specificity.

When evaluating our own findings, the following points should be stressed:

(1) In our experiments, virus- and mycoplasm-free fertile chicken eggs, inoculated before incubation, were employed;

(2) Infected groups, presenting VLPs, showed clear pathological changes: hemorrhagic perivascular infiltration, cellular damage, and a significant embryo mortality at the 7th-8th day of incubation;

(3) No pathological findings, neither biological nor morphological, were observed in the control group;

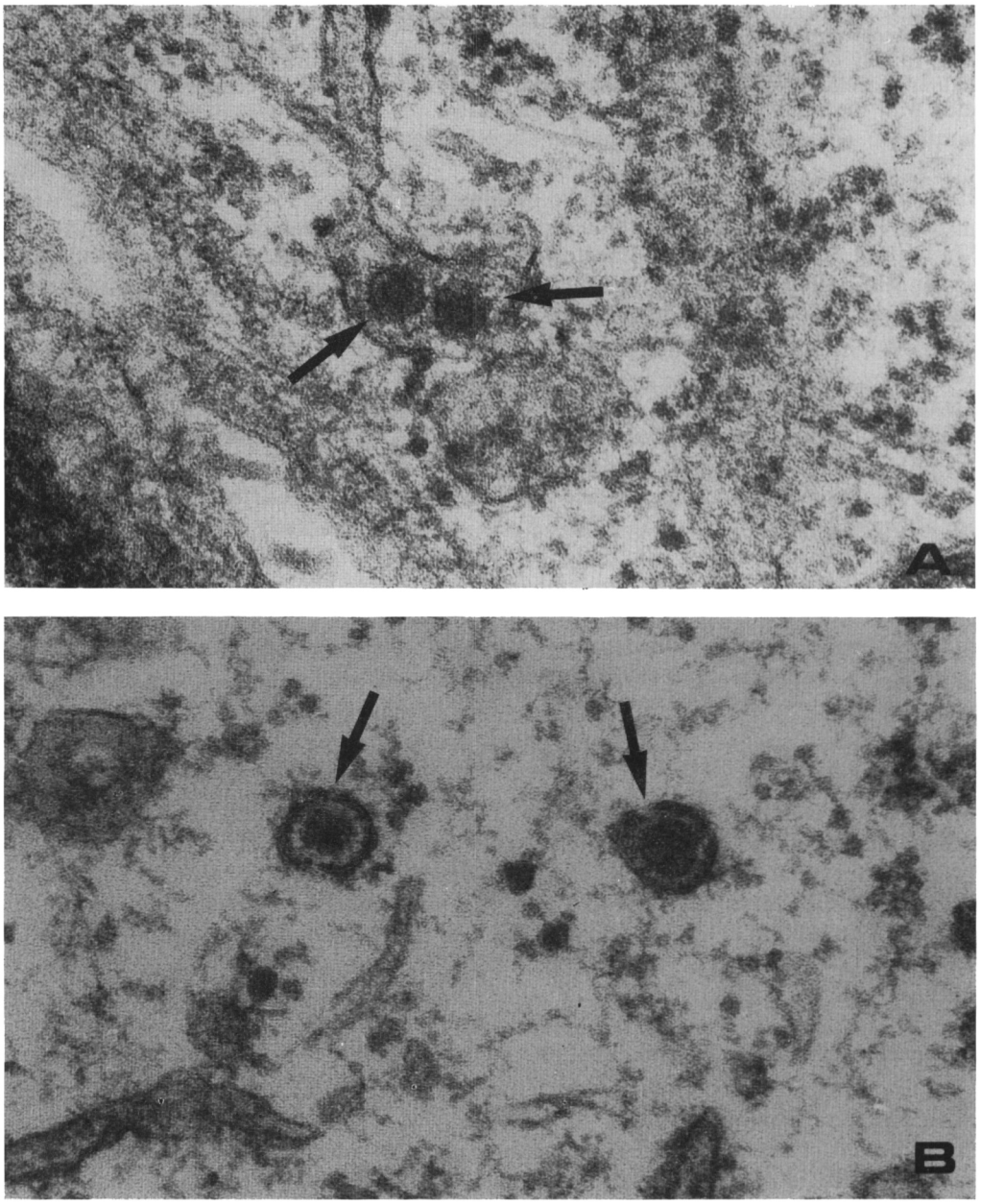

FIG. 1 A: *Chicken allantochorion in the infected group. Perivascular cell cytoplasm showing two VLPs within the rough endoplasmic reticulum.* [× 84 000].
B: *Two VLPs free in the cytoplasm of a perivascular cell of an infected chicken embryo.* [× 140 000].

https://doi.org/10.1017/S1120962300017625 Published online by Cambridge University Press

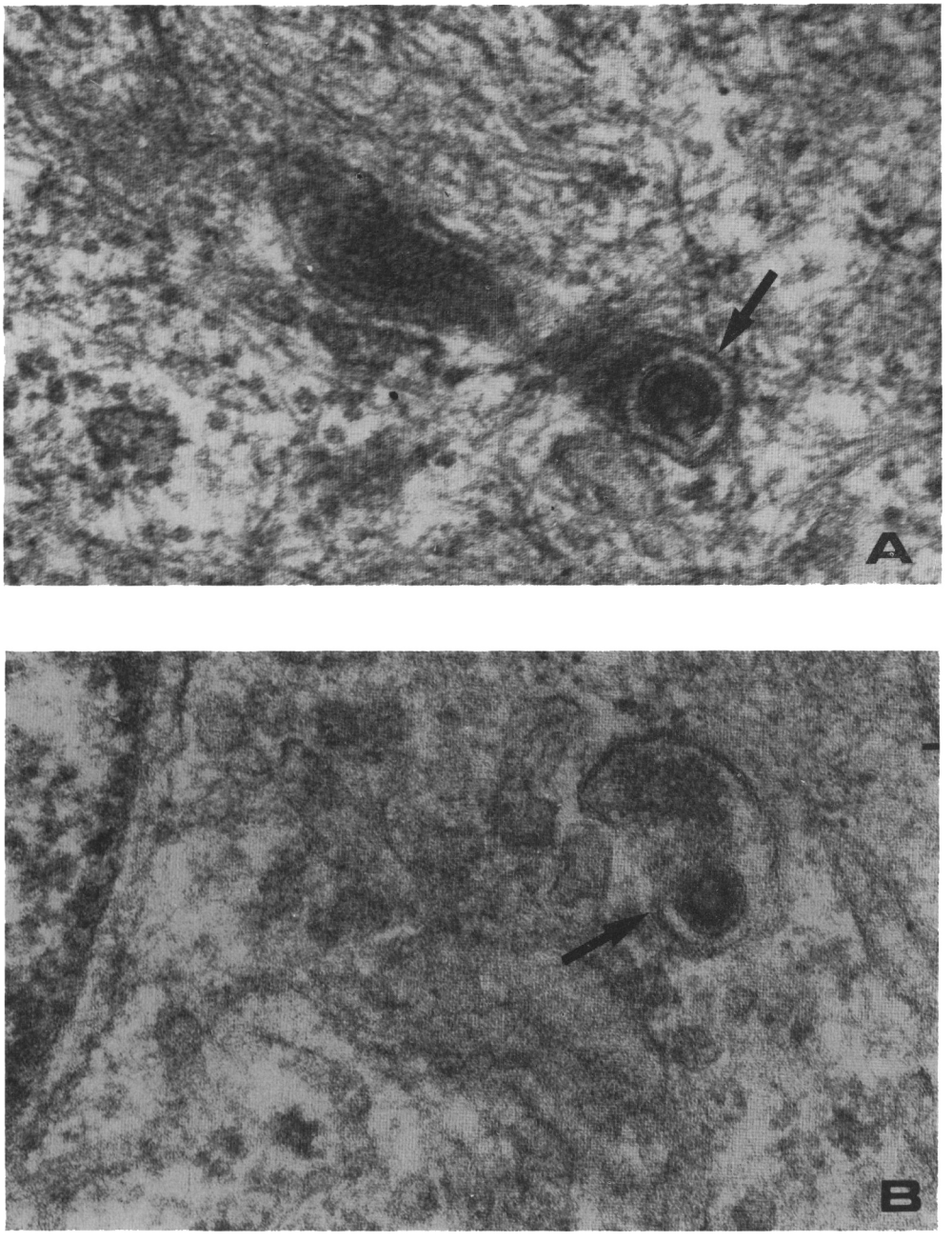

FIG. 2 A and B: *Two VLPs in the rough endoplasmic reticulum.* [× 140 000].

https://doi.org/10.1017/S1120962300017625 Published online by Cambridge University Press

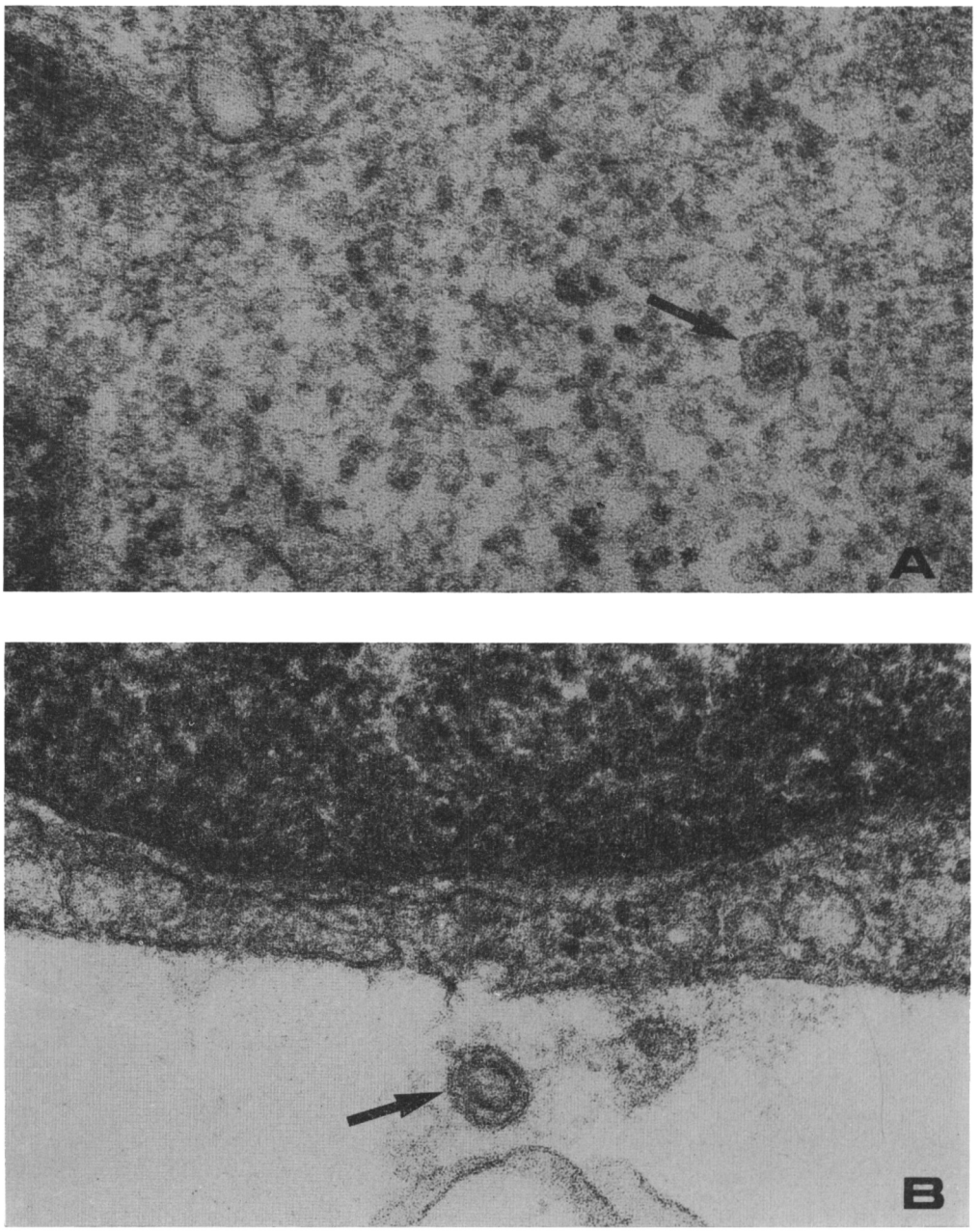

FIG. 3A: *VLP free in the cytoplasm.* [× 140 000].
B: *VLP outside the plasma membrane of a perivascular cell.* [× 140 000].

https://doi.org/10.1017/S1120962300017625 Published online by Cambridge University Press

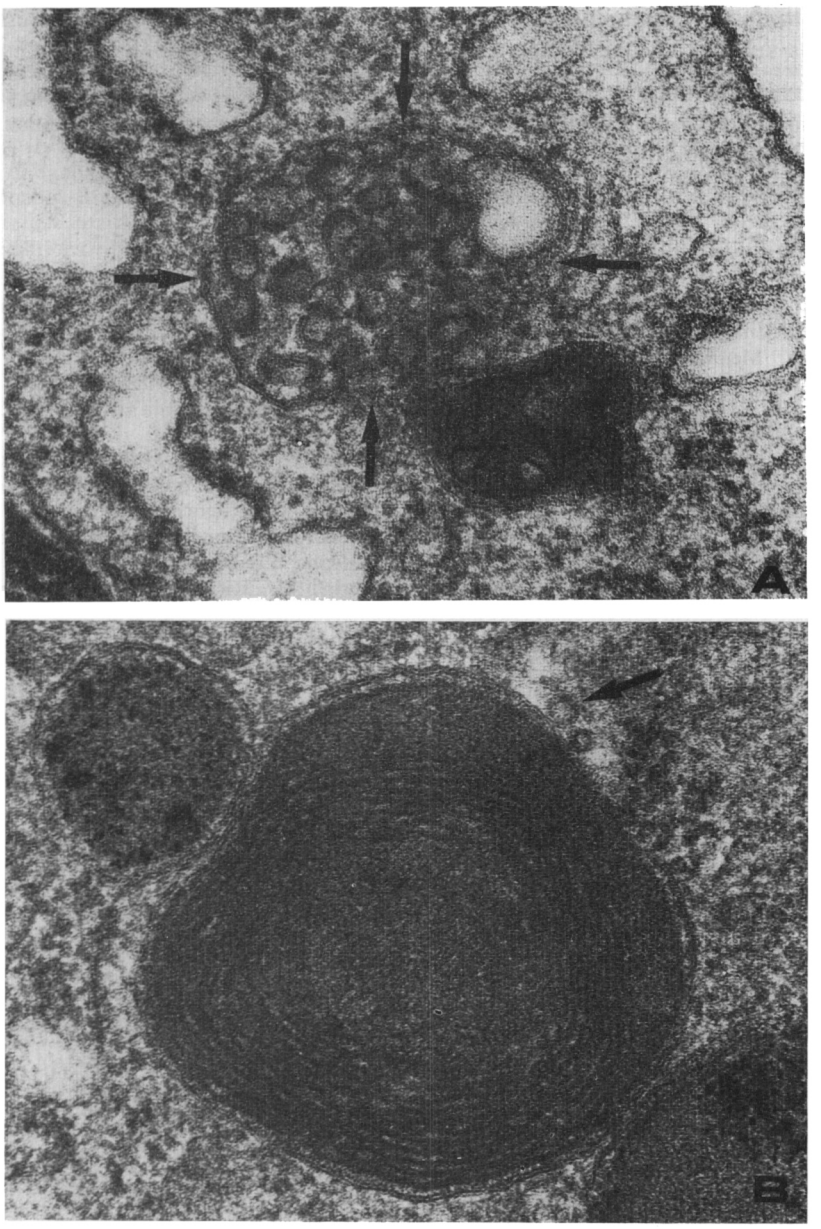

FIG. 4 A: *Group of light particles closely packed and encircled by a membrane.* [× 140 000].
B: *Detail of membranous structures frequently found in the cytoplasm under these pathological conditions.* [× 140 000].

https://doi.org/10.1017/S1120962300017625 Published online by Cambridge University Press

(4) Egg-to-egg transmission of homogenized and homogenized-ultrafiltered infected material was successfully performed.

In conclusion, it may be stated that leukemic human blood transmitted to chicken eggs produces the appearance of VLPs in the cytoplasm of the embryo's perivascular cells and a cell damage associated with its replication. Egg-to-egg transmission of VLPs has been successfully performed.

REFERENCES

Anderson D.R. 1965. Subcellular particles associated with human leukemia as seen with the electron microscope. Methodological approach to the study of leukemias. Wistar Inst. Symp. Monogr., 4: 113.

Cardinali G. 1947. Ulteriori risultati circa gli effetti ottenuti mediante l'inoculazione di materiale leucemico in uova di pollo prima dell'incubazione. Inattivazione al calore. Boll. Soc. Ital. Biol. Sper., 23: 625.

Dmochowski L., Yumoto T., Grey C.E., Designer E., Hales R.L., Langford P.L., Taylor H.G., Freireich E.J., Shullenberger C.C., Shively J.A., Howe C.D. 1967. Electron microscopic studies of human leukemia and lymphoma. Cancer, 20: 760.

Hackenbrock C.R., Rehn T.G., Weinbach E.C., Lemasters J.J. 1971. Oxidative phosphorilation and ultrastructural transformation in mitochondria in the intact ascites tumor cell. J. Cell. Biol., 51: 123.

Levine P.H., Horossewicz J.S., Grace J.T., Chai L.S., Ellison R.R., Holland J.F. 1967. Relationship between clinical status of leukemic patients and virus-like particles in their plasma. Cancer, 20: 1563.

Magrassi F., Catalano G., Coraggio F., Coto V., Cuccurullo L., D'Acunto A., Galeota G.A., Georgiades J, Nasti J.C., Violante A. 1969. Recenti acquisizioni in tema di eziopatogenesi della malattia leucemica. Relaz. " Giornate Ematologiche dell'Ospedale Maggiore di Milano ", 18-21 maggio 1969. Haematol. Lat., 12: 683.

Newell G.R., Harris W.W., Bowman K.O., Boone C.W., Anderson N.G. 1968. Evaluation of " virus-like " particles in the plasmas of 225 patients with leukemia and related diseases. N. Engl. J. Med., 278: 1185.

Porter G.H., Dalton A.J., Moloney J.B., Mitchell E.Z. 1964. Association of electron microscopic particles with human acute leukemia. J. Natl. Cancer Inst., 33: 547.

Seman G., Seman C. 1968. Electron microscopic search for virus particles in patients with leukemia and lymphoma. Cancer, 22: 1033.

Smith J.W., Freeman A.J., Pinkel D. 1967. Search for a human leukemia virus. Arch. f. Virusforsch., 22: 294.

Torrioli M., Riggio G. 1941. Reazione istioide indotta nell'embrione di pollo entro la 84ª ora di incubazione dalla inoculazione precoce di materiale leucemico. Rendiconti Scienze della Reale Accademia d'Italia, Serie VII, Vol. III.

Torrioli M., Riggio G. 1944-45. Ulteriori risultati mediante inoculazione di materiale leucemico in embrioni di pollo. Roma: Accademia Medica, Bollettini e Atti.

Torrioli M., Torrioli-Riggio G. 1947. Studi recenti sulla patogenesi delle leucemie. Clinica Nuova, 3: 153.

Torrioli M., Sensi A. 1949. Istioleucemia subacuta umana trasmessa al pollo (embrione ed adulto). Il Progresso Medico, Vol. V, N. 23.

Torrioli M., Torrioli-Riggio G. 1951*a*. Experimental researches on the pathogenesis of leukemias. Acta Haematol., 6: 361.

Torrioli M., Torrioli-Riggio G. 1951*b*. Aspects histologiques de la maladie introduite dans l'embryon de poulet par l'inoculation de matériel leucémique de l'homme. Comptes Rendus III Congrès de la Société Internationale Européenne d'Hématologie. Roma: Emes.

Torrioli M., Tentori L., Vivaldi G. 1951. Sulla filtrabilità dell'agente patogeno trasmesso all'embrione di pollo dal sangue leucemico umano. Comptes Rendus III Congrès de la Société Internationale Européenne d'Hématologie. Roma: Emes.

Torrioli M., Torrioli-Riggio G. 1953. Ulteriori contributi alla conoscenza della malattia embrionale del pollo indotta dalla inoculazione precoce di materiale leucemico umano. Nota I: Aspetti anatomo-patologici. Rendiconti dell'Istituto Su-

periore di Sanità, 16: 689.
Torrioli M., Torrioli-Riggio G., Tentori L., Vivaldi G. 1953*a*. Ulteriori contributi alla conoscenza della malattia embrionale del pollo indotta dalla inoculazione precoce di materiale leucemico umano Nota II: Dati statistici. Rendiconti dell'Istituto Superiore di Sanità, 16: 708.
Torrioli M., Torrioli-Riggio G., Tentori L., Vivaldi G. 1953*b*. Ulteriori contributi alla conoscenza della malattia embrionale del pollo indotta dalla inoculazione precoce di materiale leucemico umano. Nota III: Filtrabilità. Rendiconti dell'Istituto Superiore di Sanità, 16: 717.
Torrioli M. 1958. Embryonal chicken erythremia induced by human leukemic blood. Proc. 7th Int. Congr. Int. Soc. Haematol., p. 602. Roma: Il Pensiero Scientifico.
Torrioli M. 1963*a*. Sur l'hypothèse génético-virale dans la pathogenèse des hémoblastoses. Journal Suisse de Médecine, 41: 1463.
Torrioli M. 1963*b*. Action mutagène d'un virus dans la leucose humaine. Acta Union Internationale contre le Cancer, Vol. XIX, N. 1-2.
Torrioli M., Torrioli-Riggio G. 1964. Is human leukemia a somatic mutation of blood-forming cells due to a virus? Acta Genet. Med. Gemellol. (Roma), 13: 349.
Torrioli M., Torrioli-Riggio G., Braconi-Romei L. 1970. Communication at the 13th Int. Congr. Haematol., Munich 1970.
Torrioli-Riggio G., Cardinali G. 1951. Reazione allergica ottenuta in soggetti leucemici mediante iniezione intradermica di poltiglia di membrane corion-allantoidee di embrioni di pollo inoculati con materiale leucemico umano. Il Progresso Medico, Vol. VII, N. 10.

RIASSUNTO

L'iniezione di sangue leucemico umano in embrione di pollo prima dell'incubazione provoca una reticoloendoteliosi caratteristica che è trasmissibile da embrione a embrione ed anche al pollo adulto. L'agente virale che provoca tale reticoloendoteliosi è filtrabile e inattivabile al calore. Partendo dagli embrioni malati è possibile ottenere un antigene che provoca nella cute di leucemici una reazione nodulare. Particelle similvirali sono state fotografate con il microscopio elettronico nelle cellule degli embrioni inoculati.

RÉSUMÉ

L'injection de sang leucémique humain dans un embryon de poulet avant l'incubation produit une réticuloendothéliose caractéristique qui peut être transmise d'un embryon à l'autre et aussi bien au poulet adulte. L'agent viral qui produit cette réticuloendothéliose est filtrable et inactivable par la chaleur. Partant des embryons malades il est possible d'obtenir un antigène qui produit une réaction allergique cutanée chez les leucémiques. Des particules similvirales ont été photographiées au microscope électronique dans les cellules des embryons inoculés.

ZUSAMMENFASSUNG

Wenn man menschliches leukämisches Blut in Hühnerembryos spritzt, so tritt vor der Inkubation eine charakteristische Retikulo-Endotheliose auf, die vom Embryo aufs Embryo und auch aufs erwachsene Huhn übertragbar ist. Der virale Erreger, der diese Retikulo-Endotheliose auslöst, lässt sich filtrieren und durch Hitze inaktiv machen. Vom kranken Embryo ausgehend kann man ein Antigen gewinnen, dass auf der Haut der Leukämiker eine knötchenartige Reaktion hervorruft. In den Zellen der geimpften Hühnerembryos wurden mit dem Elektronenmikroskop virusähnliche Partikelchen bemerkt.

Prof. Mario Torrioli, Istituto Mendel, Piazza Galeno 5, 00161 Roma, Italy.