Proc. Nat. Acad. Sci. USA
Vol. 71, No. 9, pp. 3550–3554, September 1974

The Role of the Skin in Active Specific Immunization Against Leukemia in Guinea Pigs

(intradermal immunization/skin and tumor immunity/virus in guinea pig leukemia)

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ABSTRACT **The L_2C leukemia strain, which originated as spontaneous leukemia in "strain 2" guinea pigs, is transmissible by cell-graft in animals of this line; on subcutaneous inoculation it induces consistently generalized and progressive stem-cell leukemia in 99% of the inoculated animals. The leukemia thus induced never regresses. However, when very small doses of leukemic cell suspensions (0.05 ml of a 10^{-6} or 10^{-7} dilution) were inoculated intradermally, 86 out of 180 intradermal tumors (48%) regressed spontaneously. Most of the animals that recovered from the intradermal tumors were resistant to a challenging reinoculation of leukemic cells. This resistance could be substantially increased by a second intradermal inoculation of leukemic cells. Females were more resistant than males. When 55 immunized females and 36 males received a challenging subcutaneous reinoculation (0.5 ml each) of a leukemic cell suspension of 10^{-2} dilution, only two females and six males developed leukemia; the remaining 83 animals (91%) remained in good health. In a control experiment, 126 untreated "strain 2" guinea pigs were inoculated subcutaneously with the same dose, and all but one (99%) developed leukemia. The immunity thus induced could not be transferred to other animals by a serum collected from immunized guinea pigs.**

Leukemia is a progressive and fatal disease in all species, including humans. No documented evidence of permanent, spontaneous recovery from leukemia or lymphomas in humans has been reported. There have been only very rare reports of spontaneous regression of certain forms of lymphomas in chickens and, exceptionally, also in cattle. In our own studies on leukemia in mice of certain high leukemic inbred lines, such as Ak or C58, carried for more than 20 years in our laboratory, we have not observed spontaneous, permanent remission or regression of leukemia or lymphomas that developed naturally, i.e., "spontaneously," in animals of these inbred lines. We have not observed spontaneous regression of leukemia or lymphomas among the many thousands of mice or rats in which we have induced leukemia experimentally by inoculation, shortly after birth, of the mouse leukemia (Gross) virus (1).

It appears that the causative etiological agent of this disease is so potent as to prevent the affected hosts from mobilizing their defense mechanism. As a consequence, no active immunity develops in the afflicted hosts in the course of the developing disease. The pathogenic potency of the active leukemogenic agent, known to be a virus in several animal species, such as chickens, mice, rats, cats, or cattle, is so overwhelming, that it leads uniformly to the demise of the affected hosts (*1*).

It was, therefore, of great interest to observe in recent experiments carried out in our laboratory that a specific and vigorous defense mechanism could be mobilized in otherwise susceptible hosts leading to the establishment of an active, specific, and lasting immunity against the implantation of massive doses of virus-containing leukemic cells (2–4). These experiments were performed on guinea pigs of a family line uniformly susceptible to a strain of transmissible stem-cell leukemia used in this study.

MATERIALS AND METHODS

Animals. Our experiments were carried out on "strain 2" guinea pigs bred in our laboratory by brother-to-sister mating. We originally received several animals of this inbred line from Horton's Laboratory Animals Inc., Los Gatos, Calif. in 1968, and we have been breeding these guinea pigs by brother-to-sister mating in our laboratory since that time. In a few instances, F_1 hybrids born in our laboratory to Hartley females and "strain 2" males were also used and were found to be equally susceptible.

L_2C Leukemia. The L_2C leukemia strain, used in this study, originated some 18 years ago as a spontaneous leukemia in one of the "strain 2" guinea pigs bred and maintained at the National Cancer Institute. It was originally described by Congdon and Lorenz (5), and has been carried since 1954 by serial cell-graft transfer in "strain 2" guinea pigs (6). We obtained the L_2C leukemic strain in August 1968 from Dr. C. W. Jungeblut, who was then at Lenox Hill Hospital in New York City, and we have carried this strain of leukemia in our laboratory by serial cell-graft or, occasionally, by blood plasma passage.

We have observed in our studies that this strain of stem-cell leukemia is uniformly leukemogenic, on cell-graft transfer, for "strain 2" guinea pigs, and that it induces in these animals, when inoculated subcutaneously or intraperitoneally, a rapidly progressing and uniformly fatal stem-cell leukemia (7).

Preparation of Leukemic Cell Suspensions. A guinea pig with advanced leukemia was killed by ether inhalation. A fragment of the subcutaneous leukemic tumor that was removed aseptically from the site of inoculation, and also a small fragment of spleen, and of the mesenteric tumor, were weighed, cut with scissors, and ground in a mortar, sterile physiological saline solution being added to obtain a cell suspension of 10% concentration; the cell suspension was then passed through a sterile voile cloth and placed in a sterile tube immersed in a container filled with ice cubes. Serial dilutions were then prepared from the original cell suspension, and used immediately for inoculation. A cell count was made from the

TABLE 1. *Subcutaneous inoculation of leukemic cell suspensions into young, adult "strain 2" guinea pigs**

Leukemic cell dilution	No. of guinea pigs inoculated†	No. developing leukemia	Leukemia incidence (%)	Average initial latency (days)	Average latency: died from leukemia (days)
10^{-2}	126	125	99	12	19
10^{-3}	75	74	99	14	22
10^{-4}	53	51	96	21	33
10^{-5}	6	5	83	24	30
10^{-6}	11	10	91	27	33
Total	271	265	98	19	27

* Normal, healthy animals inoculated simultaneously with, and serving as controls for, those reported in Tables 2 and 3.
† 0.5 ml each.

10^{-3} dilution. Bacterial sterility was tested by inoculation of broth medium.

EXPERIMENTAL RESULTS

Induction of leukemia after subcutaneous inoculation of whole blood or of leukemic cell suspensions

In our hands, attempts to transmit the L_2C strain of leukemia by filtered extracts have not succeeded up to this date. Transmission by blood plasma inoculation was successful only when the plasma contained a few blood cells. When plasma was used after four to five successive centrifugations, 15 min each, at 3000 rpm (1400 × *g*) in a refrigerated PR-2 centrifuge, or when filtered (Selas 02) plasma was used, no leukemia was induced in the inoculated animals.

There was no difficulty in transmitting leukemia with whole blood, even in relatively high dilutions. In these experiments, with a sterile syringe containing a few drops of heparin, a few milliliters of blood were collected from a guinea pig with advanced generalized leukemia and a high peripheral blood cell count. The blood was then mixed with physiological saline solution to obtain the desired dilution. Whole blood dilutions varying from 10^{-2} –10^{-6} induced leukemia in most of the inoculated animals within 2–4 weeks after subcutaneous or intraperitoneal inoculation (0.5 ml each) into young, adult "strain 2" guinea pigs.

For routine transmission of leukemia in our experiments, we have inoculated leukemic cell suspensions prepared from leukemic tumors and spleens. Subcutaneous or intraperitoneal inoculation (0.5 ml each) of leukemic cell suspensions of 10^{-2}–10^{-6} dilution induced in practically all inoculated "strain 2" guinea pigs a progressive and fatal stem-cell leukemia.

It is apparent from Table 1, that 265 out of 271 (98%) guinea pigs inoculated with 10^{-2}–10^{-6} dilutions of leukemic cells developed and died from leukemia (Table 1). At the 10^{-6} dilution level the inoculum (0.5 ml) contained 33–520 (average 180) leukemic cells. When 10^{-7} dilutions were used for inoculation, only 3 out of 14 inoculated animals developed leukemia. However, it should be noted that at that dilution level the inoculated volume (0.5 ml) frequently did not contain more than a few leukemic cells (3–52 cells per inoculum, average 18 cells). Those animals that remained negative and did not develop leukemia were not immune; when reinoculated with higher doses of leukemic cells, they invariably developed generalized leukemia.

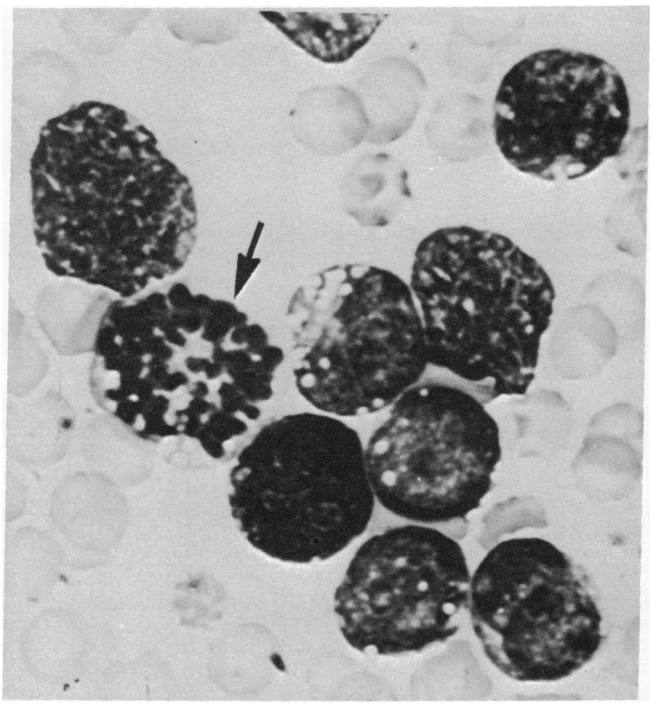

FIG. 1. Peripheral blood smear in advanced guinea pig leukemia. Very primitive stem-cells with large, hyperchromatic nuclei, coarse granular chromatin, and occasional prominent nucleoli. Cytoplasm is very scant. One mitotic figure is present (arrow). Hematoxylin and eosin. Magnification: ×1600.

In practically all animals inoculated subcutaneously with 10^{-2} –10^{-6} dilutions, a small subcutaneous nodule appeared at the site of inoculation after 2–3 weeks, occasionally a few days later.

On microscopic examination, the subcutaneous growth infiltrating the surrounding tissues had the morphology of a reticulum-cell sarcoma; the neoplastic cells resembled histiocytes; many of them had mitotic nuclei.

The subcutaneous tumors enlarged rapidly. Within about 1 week after their appearance, metastatic tumors developed in the inguinal and axillary lymph nodes; at the same time, the peripheral blood began to show pathologic changes; the number of leukocytes increased and blast cells appeared (Fig. 1). The number of leukocytes increased rapidly in the terminal stages, reaching up to 400,000 leukocytes/mm³. The increase of leukocytes in peripheral blood with appearance of blast cells was observed consistently in all cases of leukemia in our animals.

Examination of leukemic guinea pigs killed in terminal phases revealed the presence of subcutaneous tumors at the site of inoculation, usually also of metastatic lymph nodes in the axillary and inguinal areas, and also of large mesenteric tumors; the spleen was in all instances considerably enlarged and friable; the liver was also enlarged. On microscopic examination, all organs examined were found to be infiltrated with leukemic cells. *The thymic lobes, which are located on both sides of the neck in guinea pigs,* were also infiltrated with leukemic cells. It should be noted, however, that the involvement of the thymus in guinea pigs was much less pronounced than that usually observed in mice and rats.

TABLE 2. *Intradermal inoculations of small doses of leukemic cell suspensions into young, adult "strain 2" guinea pigs*

Sex	Leukemic cell dilution	No. of guinea pigs inoculated*	No. developing i.d. tumors	Average time i.d. tumors developed (days)	No. i.d. tumors regressed	Incidence i.d. tumors regressed (%)	No. developing generalized leukemia after i.d. tumors regressed	Total developing generalized leukemia
♀ ♀	10^{-4}	106	97	14	43	44	0	55†
♂ ♂	10^{-4}	58	56	15	24	43	0	32
♀ ♀	10^{-6}	132	94	19	48	51	9	55
♂ ♂	10^{-6}	83	55	18	20	36	0	35
♀ ♀	10^{-7}	34	13	22	8	62	0	5
♂ ♂	10^{-7}	77	18	20	10	56	0	8
Total ♀ ♀ (10^{-6} & 10^{-7})		166	107	21	56	52	9	60
Total ♂ ♂ (10^{-6} & 10^{-7})		160	73	19	30	41	0	43
Grand total (10^{-6} & 10^{-7})		326	180	20	86	48	9	103

i.d., intradermal.

* 0.05 ml each, inoculated intradermally.

† One guinea pig in this group died from generalized leukemia 45 days after intradermal inoculation with no preceding development of an intradermal tumor.

Similar results were observed after intramuscular or intraperitoneal inoculations of leukemic cells, except that the intraperitoneal route was slightly more sensitive, leading more rapidly to generalized leukemia.

Virus Particles in the Tumor Cells. Electron microscopic examination done by Dorothy G. Feldman in our laboratory (8) revealed that the histiocytic-like tumor cells contained large numbers of spherical doughnut-like virus particles about 90 nm in diameter, having a thick, granular, rather fuzzy outer coat. Immature virus particles with electron-lucent centers and two concentric shells were observed budding from the membranes or free within cisternae of the endoplasmic reticulum. In addition, mature virus particles with electron dense nucleoids and single, thick outer shells were found mainly in the intercellular spaces.

Intradermal inoculation of leukemic cells

Subcutaneous inoculation of leukemic cells led consistently to the development of generalized leukemia. On the other hand, intradermal inoculation of very small doses of leukemic cell suspensions led to the development of intracutaneous tumors that either grew progressively or that regressed spontaneously after a temporary growth.

The results of intradermal inoculations depended essentially on the technique used and the number of leukemic cells inoculated. The volume inoculated was about 0.05 ml. The dilutions used for inoculation varied from 10^{-4}-10^{-1}. In the majority of experiments, 10^{-6} and 10^{-7} dilutions were used. At the 10^{-6} dilution level, the number of leukemic cells in 0.05 ml varied from 3–50 (average 18 cells). At the 10^{-7} dilution level, the number of cells in 0.05 ml volume varied from 0–5 (average two cells).

The technique of intradermal inoculation was of primary importance. In a small area on the flank of the guinea pig the hair was clipped closely with an electric clipper and the skin was lightly sponged with 70% ethyl alcohol. The leukemic cell suspension was then inoculated very carefully into the upper layer of the skin, with a sharp 26-gauge needle. Complete penetration of the skin had to be avoided to prevent the introduction of leukemic cells into the subcutaneous tissues. Immediately after inoculation, the skin puncture was covered with a drop of collodion to prevent reflux of the inoculated cell suspension. If the intradermal inoculation was well made, a small epidermal wheal appeared at the site of inoculation.

Results of intradermal inoculations

Guinea pigs inoculated intradermally with small doses of leukemic cell suspensions could be essentially divided into the following three groups: The first group represented those guinea pigs in which the induced intradermal tumors grew progressively and led to the development of a generalized leukemia.

In animals of the second and most important group, the induced intradermal tumors persisted only temporarily and eventually regressed without trace. Most of the animals in which the tumors regressed were resistant to reinoculation of massive doses of leukemic cells.

The third group consisted of those guinea pigs that did not develop any tumors at the site of intradermal inoculation and remained apparently in good health.

Progressively Growing Intradermal Tumors. Over 50% of all intradermal tumors induced with 10^{-4} dilutions of leukemic cells, and less than 50% of those induced with higher dilutions, grew progressively and led to generalized leukemia (Table 2). Most of the inoculated animals developed intradermal tumors at the site of inoculation within 3 weeks; in those animals in which the induced tumors grew progressively, metastatic tumors appeared 2–3 weeks later in the axillary and inguinal lymph nodes, gradually leading to generalized leukemia involving the mesenteric lymph nodes, spleen, liver, and other organs, as well as bone marrow and peripheral blood.

Intradermal Tumors that Lasted Only Temporarily and Later Regressed. In 86 out of 180 animals (48%) that developed intradermal tumors at the site of inoculation, the tumors grew

only temporarily and gradually regressed (Table 2). The incidence of regression was higher in animals that received the 10^{-6} -10^{-7} dilutions, as compared with those that received 10^{-4} dilutions of leukemic cell suspensions. The intradermal tumors appeared at the site of inoculation after 11–26 days (average 18–20 days), and gradually increased in size. Those intradermal tumors that eventually regressed remained very small and lasted usually about 6–9 days, then gradually regressed without trace. Some of the very small intradermal tumors regressed very promptly, within only a few days; on the other hand, in rare instances they lasted up to 14 days before they regressed.

When examined with the optical and electron microscope, cell necrosis was a conspicuous feature of the regressing intradermal tumors and was manifested by large numbers of macrophages with engulfed lysed cells or cell fragments. The virus particles disappeared before the tumors regressed (9).

There was an interesting sex difference in the incidence of regression of the induced intradermal tumors. This was apparent when 10^{-6} and 10^{-7} dilutions were inoculated. It is evident from Table 2 that 52% of the intradermal tumors regressed in females, as compared with only 41% in males. The difference between males and females in the incidence of spontaneous regression of the induced intradermal tumors could be readily abolished by a higher dose of the inoculated cells, since no sex difference in the incidence of regression of the intradermal tumors was observed when 10^{-4} dilutions were inoculated.

Among 153 guinea pigs in which the intradermal tumors regressed (Table 2), nine guinea pigs developed generalized leukemia 40–70 days after the spontaneous disappearance of the intradermal tumors and one guinea pig died from generalized leukemia, apparently with no preceding development of an intradermal tumor. Similar observations were made in our previous studies in which the intradermal tumors were induced in guinea pigs by scarification of skin with leukemic cell suspensions (4).

Resistance of guinea pigs in which the intradermal tumors regressed to reinoculation of leukemic cells

The majority of guinea pigs in which the intradermal tumors regressed were resistant to reinoculation of heavy challenging doses of leukemic cells. This resistance could be substantially increased by a second intradermal booster inoculation of leukemic cells (0.05–0.1 ml of a 10^{-3} or 10^{-4} dilution of leukemic cell suspension).

Animals in which the intradermal tumors regressed spontaneously and which received a second intradermal inoculation of leukemic cells were solidly immunized. Out of a total of 91 immunized guinea pigs challenged by subcutaneous inoculations of massive doses (0.5 ml each) of leukemic cell suspensions of 10^{-2} dilution, only seven animals (8%) developed leukemia (Table 3). Females were more resistant than males. Out of 55 females that received subcutaneous challenging inoculations, only one developed leukemia after 2–3 weeks. A second female in this group developed leukemia after a long latency of 9 months. Thirty-six immunized males were challenged subcutaneously with a similar dose of leukemic cells, and six of them developed leukemia 15–33 days after the subcutaneous challenge.

In a control experiment, 126 young, adult, healthy "strain 2" guinea pigs were inoculated subcutaneously with the same dose (0.5 ml of a 10^{-2} dilution) of leukemic cells, and all but one (99%) developed generalized leukemia within 2–3 weeks after inoculation (Table 1). In an additional control experiment, eight normal healthy guinea pigs were inoculated subcutaneously (0.1 ml each) with a 10^{-6} dilution of a leukemic cell suspension (average 36 cells per inoculum) and all developed generalized leukemia after 3–4 weeks.

TABLE 3. *Resistance of immunized* "strain 2" guinea pigs to subcutaneous reinoculation of leukemic cells*

Sex	No. of guinea pigs challenged by s.c. inoculation†	No. of guinea pigs that developed leukemia	Incidence of leukemia developed after s.c. challenge (%)	Average latency: died from leukemia after s.c. challenge (days)
Females	55	1‡	2	14
Males	36	6	17	29
Total	91	7	8	27

s.c., subcutaneous.

* Initial intradermal immunization followed by intradermal booster inoculation.

† 0.5 ml of leukemic cell suspension of 10^{-2} dilution, inoculated subcutaneously.

‡ One additional female developed leukemia after a long latency of 9 months. Not included in this table.

The challenging subcutaneous inoculation that was usually used (0.5 ml of a 10^{-2} dilution) contained an average of 1,800,000 leukemic cells; this very large dose, which was tolerated by over 90% of the immunized guinea pigs, would have been sufficient to induce generalized leukemia in at least 50,000 untreated "strain 2" guinea pigs.

Animals that did not react to intradermal inoculation of leukemic cells

Among 326 guinea pigs inoculated intradermally with leukemic cell suspensions, 137 did not develop intradermal tumors and remained in good health (Table 2). One hundred and seven animals of this group were reinoculated intradermally (0.05 ml each) with leukemic cell suspensions of 10^{-4} dilution, and 66 of them developed intradermal tumors at the site of the second intradermal inoculation; 25 of these tumors later regressed (38%). Six animals developed generalized leukemia after the intradermal tumors regressed, and three other guinea pigs developed leukemia without any cutaneous tumors developing at the site of either the first or the second intradermal inoculation. Thus, a total of 69 out of 107 reinoculated animals (64%) developed either intradermal leukemic tumors or generalized leukemia. These results clearly suggest that the majority of those guinea pigs that did not apparently react to the initial intradermal inoculation remained susceptible to a reinoculation of leukemic cells. It is reasonable to assume that these guinea pigs did not receive, or did not retain, a sufficient number of leukemic cells in the course of the initial intradermal inoculation, since part of the inoculum might have been expelled through the skin puncture; as a result, such guinea pigs remained susceptible. Another small group representing about one-third of the total among those that apparently did not react to the initial intradermal inoculation developed immunity, even though in these animals the development and subsequent regression of intradermal tumors

had not been recorded. It is possible to assume, nevertheless, that in guinea pigs of this group the initial intradermal inoculation of leukemic cell suspensions did actually result in the formation of very small leukemic tumors that remained unnoticed and subsequently regressed. Recognition of very small intradermal tumors that develop after intradermal inoculation of small doses of leukemic cell suspensions may be quite difficult. Such intracutaneous tumors may be barely visible. We have noticed that some of the induced intradermal tumors were so small as to represent only a slightly elevated, round thickening of the skin, about 2 or 3 mm in diameter. Some of these very small tumors disappeared without trace after 2–3 days. Such animals, however, were then immune to reinoculation of leukemic cells.

Intradermal inoculation of inactivated leukemic cells or filtrates

In previous studies we have determined that immunity could not be induced by intradermal inoculation of either heated (56°, 0.5 hr) leukemic cell suspensions or extracts prepared from normal guinea pig organs (10).

In our current study we have inoculated six guinea pigs intradermally with leukemic cell suspensions mixed (1:500) with formaldehyde solution, followed by a second similar intradermal booster inoculation. Three of these guinea pigs were then challenged by subcutaneous inoculation (0.5 ml) of a 10^{-2} leukemic cell suspension, and all three developed leukemia. The remaining three received, as a challenging inoculation, an intradermal inoculation (0.05 ml each) of a 10^{-3} dilution of leukemic cells. As a result, 2 of them developed progressively growing intradermal tumors leading to generalized leukemia, and one developed a transient intradermal tumor that regressed spontaneously.

In another series, seven guinea pigs were inoculated intradermally with filtrates (Selas 02) prepared from leukemic cell extracts, in order to determine whether intradermal inoculation of virus particles without leukemic cells could induce immunity; a second intradermal booster inoculation of a leukemic filtrate was made after 3 weeks. The seven guinea pigs were then inoculated subcutaneously with a challenging dose (0.5 ml each) of a leukemic cell suspension (10^{-2}), and all developed generalized leukemia 3–4 weeks after the challenging inoculation.

CONCLUSIONS

Experiments reported here suggest that a solid, specific immunity against experimental leukemia can be induced in guinea pigs. The method of immunization consists of intradermal inoculation of very small doses of leukemic cell suspensions. This inoculation induces a small intradermal tumor at the site of inoculation; some of the induced tumors grow progressively and lead to a generalized leukemia; about 50% of the induced intradermal tumors, however, regress spontaneously. Those animals in which the intradermal tumors regressed are immune to reinoculation of leukemic cells. The immunity can be increased by a second intradermal booster inoculation of leukemic cells, a method essentially similar to a booster reinoculation of vaccines, routinely used in methods of immunization against many bacterial and viral diseases. Females are more resistant than males. Most of the immunized animals tolerate with impunity a massive challenging reinoculation of leukemic cells.

These results demonstrate that active and specific immunity can be induced against leukemia in mammals, and suggest that the skin plays a very important role in the induction of immunity against leukemia and presumably also against certain other neoplastic diseases. It is obvious, however, that at the present time this method has only a purely theoretical significance and has no practical application in its present form. Many animals die in the course of immunization or may develop leukemia, as a delayed reaction, several months after the initially induced intradermal tumors regressed.

Up to the present time we have not been able to transmit the induced immunity to other animals by a serum collected from immunized guinea pigs (3). It appears that immunity acquired against leukemia is not humoral, but is closely related to the cells, from which it cannot be separated under currently applied experimental conditions.

We are grateful to Dr. Theodore Ehrenreich for reviewing the microscopic slides and providing the photomicrograph, and to Miss Lorraine A. Moore for excellent technical assistance. This study was supported, in part, by a grant from the Chemotherapy Foundation of New York.

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