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Abstract

Spontaneous C3Hf lymphatic leukemia maintained in an ascites form was transplanted into newborn Wistar rats less than 24 hours old. Single subcutaneous or intraperitoneal inoculation of the neoplastic cells resulted in progressive tumor growth fatal to the heterologous hosts. Limited serial passage through newborn rats was performed. The intraperitoneally heterografted leukemia grew as massive lymphosarcoma predominantly in the adipose and connective tissue compartments with invasion of the neighboring organs but without leukemic manifestations. The characteristic behavior and histopathologic features of the transplanted disease are presented in comparison with the results of similar experiments reported in the literature.

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STUDIES ON LEUKEMIA IN THE C3Hf STRAIN OF MICE II. HETEROLOGOUS TRANSPLANTATION OF MOUSE LEUKEMIA INTO NEWBORN WISTAR RATS

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Heterologous transplantation of tumors constitutes an important aspect of experimental cancer research. Because of still obscure immune mechanisms, untreated adult animals do not accept normal or neoplastic tissues from foreign species. This heterograft rejection can be partially or completely circumvented by the use of embryos[1,2] or newborn animals,[3-7] by prior conditioning of host animals with X-irradiation and/or cortisone,[8-12] or by transplanting tumor cells into certain protected sites such as the anterior chamber of the eye,[13-15] brain[16-18] or cheek pouch.[19-24]

Heterotransplantation of murine leukemias into newborn rats was carried out by THOMPSON and GURNEY,[5,6] and KIRSTEN *et al.*[7] HANDLER[22] succeeded in serial passage of a mouse lymphoma in the hamster cheek pouches.

Human malignant lymphomas failed to grow progressively in irradiated rats,[25] in the anterior chambers of the eyes of guinea pigs,[15,26] or in the cheek pouches of cortisone-treated hamsters.[21,23]

The present communication deals with experiments in which spontaneous lymphatic leukemia maintained as an ascites form in C3Hf strain mice was transplanted into newborn Wister rats and its behavior and morphology in these heterologous hosts were studied.

MATERIALS AND METHODS

Transplantable mouse leukemia: The mouse leukemia used for heterotransplantation is a lymphatic leukemia that arose spontaneously in a low-leukemic strain C3Hf mouse and has been serially passaged in an ascites form in this laboratory. As reported in the previous paper,[27] the leukemia is a rapidly growing tumor, regularly killing the hosts in 9～10 days following intraperitoneal inoculation of the ascitic fluid. By the time the inoculated mice succumb to generalized leukemia, there occurs an accumulation of 1～2 ml. of milky ascitic fluid, containing approximately 5×10^5 tumor cells per cu. mm. This leukemia line has shown a strict strain specificity and is transplantable only to mice of the C3Hf strain in which the leukemia originated.

Animals: The C3Hf mice used were inbred in our mouse colony by brother-to-sister mating from a nucleus obtained in 1958 from the Baylor University College of Medicine, Houston, Texas. Pregnant random-bred Wistar rats were purchased from a commercial breeder in Kobe, Japan. They were closely observed for delivery and newborn rats were used within 24 hours after birth. All the animals were housed in wooden boxes on wood shavings, and fed Oriental laboratory chow in pellets and water *ad libitum*.

Transplantation: Freshly drawn ascitic fluid obtained from the donor mice was injected into newborn rats. Prior to inoculation, the number of tumor cells in the ascitic fluid was counted in a Neubauer hemocytometer. Intraperitoneal inoculation of the ascitic fluid gave rise to a solid tumor in some of the transplanted rats. For subtransplantation, part of the solid tumor was snipped into small pieces and gently homogenized in a glass homogenizer with addition of physiological saline solution. The cell suspension was passed through a fine stainless steel mesh to remove coarse particles, and the number of viable cells were counted according to the method of Schrek.[28] The procedure included the following steps: 0.2 ml. of the cell suspension was added to 4.8 ml. of a 1 : 2000 eosin solution in Tyrode's at pH 7.6 and shaken for two minutes. A drop of this mixture was placed in the hemocytometer. Unstained intact cells were slightly yellowish and stained cells were diffusely pink. Only the unstained cells were counted and the number of viable tumor cells per cu. mm. calculated.

Newborn Wistar rats less than 24 hours of age were inoculated either intraperitoneally or subcutaneouly with the ascitic fluid or tumor cell suspension. The inoculums ranged from 0.1 to 0.2 ml. per animal, and all the injections were performed through a fine (1/3 gauge) needle attached to a tuberculin syringe. To prevent leakage of fluid, the needle was inserted into the lower abdominal cavity through the upper thigh. Immediately after the inoculation, the litters were returned to their respective mothers.

Experimental analysis: The animals were inspected daily and any change in the growth characteristics was recorded. Postmortem examination was performed on all rats which died or appeared moribund with marked swelling of the abdomen. Those rats rejecting the heterotransplants have remained apparently healthy and are now under the continued observation.

At the time of autopsy, the peripheral leukocytes were counted and blood smears were made. In addition, fragments of the hematopoietic organs were suspended in a drop of horse serum on slides and smeared. All the smears were stained with May-Giemsa stain. Histologic sections were taken from the representative organs of each experimental animal. Tissues were fixed in 10% formalin, processed routinely and stained with hematoxylin and eosin.

RESULTS

The results of heterologous transplantation of C3Hf leukemia cells into newborn Wistar rats are shown in Tables I and II. The animals received either a single intraperitoneal or subcutaneous inoculation at birth. The neonatal mortality rate due to the treatment was minimal. Inoculations of the leukemic cells into young adult Wistar rats by the same routes were unsuccessful.

Intraperitoneal inoculation; The C3Hf ascites tumor, when first trans-

Table I. Susceptibility of Newborn Wistar Rats to Inoculation with C3Hf Ascites Leukemia Cells

Route of inoculation*	No. cells injected ($\times 10^6$)	No. donors	No. litters injected	No. newborns injected	Lymphoma ratio+	Latency in days
i. p.	50	1	1	8	2/7 (29%)	14～16
i. p.	60.5	1	1	12	6/12(50%)	15～24
s. c.	80.6	1	1	10	2/9 (22%)‡	33

* i. p.: intraperitoneal. s. c.: subcutaneous.
\+ ratio: with lymphoma/rats surviving neonatal injection.
‡ regression in one of them.

Table II. Subtransplantation of C3Hf Leukemia through Newborn Wistar Rats*

Transpl. generation	No. donors	No. litters injected	No. newborns injected	No. cells injected ($\times 10^6$)	Lymphoma ratio+	Latency in days
1	2	2	20	50～60.5	8/19(42%)	14～24
2	1	1	7	16	1/7 (14%)	18
3	1	1	9	19.7	0/9	

* intraperitoneal route only.
\+ ratio: rats with lymphoma/rats surviving neonatal injection.

ferred from mouse to rat, was in the 29th transplant generation. Two litters consisting of 20 newborns were inoculated with 0.1 ml. ($50\sim60.5\times10^6$ cells) of ascitic fluid harvested from two donor mice. Progressive tumor growth resulted in 8 (42 %) of 19 surviving rats. The intervals between inoculation and death of these eight rats ranged from 14 to 24 days (Table II). The percentage of positive takes differed in the two litters given inoculation intraperitoneally with different cell doses (Table I).

About 10 days after the intraperitoneal inoculation, the rats in which positive takes had occurred began to show abdominal swelling, which progessed to the death of the hosts over the next 1～2 weeks. Emaciation, retarded growth and sparse hair characterized the tumor-bearing rats. In no instances regression of the abdominal tumor followed.

Postmortem examination of these rats revealed massive tumor growth completely filling the abdomioal cavity (Fig. 1). Grayish white, friable neoplastic tissue was seen infiltrating the omentum, mesentery, hepatic hilum, and periureteral fat. There was in both flanks an oval discrete tumor nodule, 1～2 cm. in diameter, which was connected with a narrow pedicle to the retroperitoneal tumor (Fig. 2). Central necrosis of the tumors was slight and infrequent. The liver was of normal size and yellowish in color with an accentuation of the lobular

markings. Sections through the liver showed parenchymal invasion by the neoplastic growth, extending from the *porta hepatis* to the peripheral region. In advanced cases, more than one half of the entire liver was replaced by the tumor. No ascitic fluid was demonstrated in any of the animals examined. The thymus and spleen appeared normal in size and the lymph nodes were not enlarged.

Peripheral leukocyte counts made at the time of autopsy were between 10,000 and 30,000 per cu. mm., and there were no leukemic cells in the blood. Cell suspension smears of spleen disclosed occasional tumor cells, but those of thymus and bone marrow contained no such cells.

Histologically, the intraabdominal tumor was lymphosarcoma composed of immature lymphoid cells with scanty cytoplasm and vesicular nuclei (Fig. 4). Mitotic figures were numerous. The liver was involved in all the eight rats by direct invasion of the neoplastic cells whose portal of entry appeared to be the hilar area. Its parenchyma was replaced by a sheet of tumor cells among which more resistant bile ducts were seen remaining (Fig. 5). Another notable feature was marked fatty metamorphosis of the liver. It was centrolobular in distribution; the liver cells around the central vein were enlarged and loaded with fat droplets, and dilatation of the central vein was often noted (Fig. 6). Most of the fat-loaded cells had appearently intact nuclei and degenerative changes were rare. Several rats also showed invasion of the stomach (Fig. 7), pancreas, and adrenal by the tumor cells. Some of them, in addition, showed tumor encroachment of the kidney, ureter, and retroperitoneal muscle. The pancreas (Fig. 8) and adrenal were heavily infiltrated and largely replaced by the tumor cells. The kidney invasion was slight and was located in the very external cortex under the disappearing capsule and perivascularly (Fig. 9). Sections of spleen revealed no significant structural alterations. Thoracic organs were normal both grossly and histologically.

Histological findings as well as cytological features of the neoplastic cells grown in the rats were indistinguishable from those of tumor cells maintained in mice of the C3Hf strain (Fig. 3).

Serial transplantation : An attempt was made to carry the mouse tumor serially through the newborn rat. A tumor cell suspension was made from the first rat-generation transplant according to the technic described under the Materials and Methods. Seven newborn rats were inoculated intraperitoneally with 0.1 ml. (16×10^6 cells) of the cell suspension and one (14%) of them died of abdominal lymphosarcoma after 18 days (Table II).

Gross pathology of this rat was identical with that of rats dying with the first heterotransplants. Histologic examination revealed invasion of tumor cells in most of the abdominal organs including the liver, stomach, pancreas, kidney,

ureter, small intestine, parietal peritoneum, and retroperitoneal muscle. Infiltration in these organs was again due to the contiguous extension of local growth of the inoculated cells aud not metastatic as is often seen in leukemic conditions.

A tumor cell suspension was further prepared from this rat with the second-generation transplant, and 0.15 ml. (19.7×10^6 cells) of the suspension was injected intraperitoneally into 9 newborn rats. None of them, however, developed positive growth (Table II).

Subcutaneous inoculation: The C3Hf ascites tumor in the 33rd transplant generation was used for subcutaneous inoculation. Ten newborn rats were injected subcutaneously with 0.15 ml. (80.6×10^6 cells) of the ascitic fluid and 2 (22%) of the 9 surviving rats developed subcutaneous masses (Table I). In one of them, the tumor grew progressively and killed the host in 33 days after the inoculation. But in the other rat, the subcutaneous mass, after having reached a considerable size, completely regressed by 33 days. Because of the cannibalism, autopsy data are not available on the tumor-bearing rat.

Double intraperitoneal inoculations. —A litter of 10 newborn rats was inoculated with 0.1 ml. (26.5×10^6 cells) of a tumor cell suspension prepared from a rat with the first rat-generation transplant. After 10 days, there were no signs of positive takes in any of the 9 surviving rats. And so one of them was sacrificed in order to ascertain the absence of tumor cells and to study the histology of liver of a non-tumor-bearing rat. Both grossly and microscopically the rat was normal, and the liver was devoid of fatty metamorphosis. On the same day, the remaining 8 rats were injected intraperitoneally with 0.2 ml. (37.9×10^6 cells) of a cell suspension prepared from enlarged spleens and mesenteric lymph nodes of two C3Hf mice with leukemia resulting from back-transplantation of the first rat-generation transplant. To date, for two months, all of them have remained apparently healthy without any sign of tumor growth.

Back-transplantation from rat to mouse: Two adult C3Hf mice were transplanted intraperitoneally with 0.2 ml. (53×10^6 cells) of a tumor cell suspension prepared from the first rat-generation transplant. Both of them developed the usual picture of transplanted leukemia with milky ascites after 10 days, when they were used as donors for the experiment described above.

DISCUSSION

The C3Hf ascites leukemia cells, when transferred to newborn Wistar rats, resulted in the development of intraperitoneal lymphosarcoma invading the neighboring abdominal organs. However, the heterotransplants remained localized in the abdomen without generalization or leukemic blood pictures. This is in sharp contrast to the situation of isologous transplantation of the leukemia, in which leukemic infiltration of most of the distant organs and terminal high

leukocyte counts were the rule.[27] Enlargement of the spleen and mesenteric lymph node, a constant feature of the transplanted C3Hf mice, was no longer observable in the rats. Instead, the neoplastic cells showed a particular preference for the adipose and connective tissues, and completely replaced the omentum, mesentery, peripanceratic and perirenal fat with frequent contiguous invasion of the liver, stomach, pancreas, and adrenal. Invasion of other abdominal organs such as the kidney, small intestine, and spleen was much less infrquent than was expected from the gross appearance.

Although occasional tumor cells were found in the smears of rat spleen, apparently they failed to proliferate vigorously to cause splenomegaly in the heterologous environment. All these findings indicate that newborn rats, because of their immunological incompetency, allow local growth of the alien neoplastic cells but they are not entirely defenseless as to permit them to metastasize to remote organs.

THOMPSON and GURNEY[5] obtained a localized growth in Wistar rats inoculated subcutaneously, when newborn, with mouse lymphoma P1534 cells. Serial passage of this tumor in the heterologous hosts was unsuccessful.[6] They also reported that two other rapidly growing, ascites-forming mouse lymphomas 6C3HED and L4946 were serially transplantable through newborn rats.[6] It is not stated, however, whether thery grew as a localized tumor or became generalized in the heterologous hosts.

ERDMANN *et al.*[29] reported that a mouse lymphoma L1210 always regressed if inoculated into newborn and adult rats of the Sprague-Dawley strain, whether they were conditioned or not.

KIRSTEN *et al.*,[7] on the other hand, transferred spontaneous viral leukemia of the AKR strain into newborn Wistar rats. In their experiments, single subcutaneous or intraperitoneal injections of leukemic cell suspensions did not result in progressive tumor growth, and two intraperitoneal injections with an interval of 7～10 days were neccesary to establish positive heterografts. In contrast to the present observation and that of THOMPSON and GURNEY,[5] the heterotransplanted AKR leukemia assumed the growth pattern of a generalized leukemic process with high leukocyte counts in the peripheral blood.[7]

HANDLER[22] transplanted a mouse lymphoma P1534, presumably the same tumor as that employed by THOMPSON and GURNEY,[5,6] into the cheek pouches of untreated, young adult hamsters, and observed the development of few generalized leukemia as well as localized tumors during its serial passage in the heterologous hosts.

It is thus apparent from these divergent observations that the malignancy of cells, route of inoculation, and age and genetic background of the host animal determine the fate of mouse leukemias in heterologous transplantation.

THOMPSON and GURNEY,[6] in the course of their experiments, noted the occurrence of runt disease in nearly all newborn rats inoculated with various mouse tumors. KIRSTEN *et al.*[7] also observed a similar disease in newborn rats given single intravenous injection of normal AKR cells. The disease was characterized by retarded growth, sparse hair, diarrhea, and depletion of lymphoid elements in the thymus, spleen, and lymph nodes. In the present experiments, there was no increased mortality directly ascribable to immunologic runts authenticated by the histologic evidence, although retarded growth and sparse hair attended in some of the tumor-bearing rats.

The C3Hf mouse leukemia could not be serially passaged through newborn rats, apparently because the cell dosage used in the second and third transfers was too small. With a larger number of leukemic cells however, it would have been possible to establish permanent heterografts.

The method of inducing acquired immunological tolerance described by BILLINGHAM *et al.*[30] found its application in the homotransplantation experiments of KOPROWSKI,[31,32] who succeeded in transplanting the 6C3HED ascites tumor into Swiss mice pre-treated, during their embryonic life, by intrauterine injection of whole blood from C3H mice. The experiments of KIRSTEN *et al.*[7] are comparable to these experiments, although the first injection was given within 24 hours after birth and the interval between the first and challenge injection was much shorter. In an experiment reported herein, two intraperitoneal inoculations, spaced 10 days apart, were given to eight newborn rats without success. THOMPSON and GURNEY,[6] and KOBAYASHI,[33] likewise, observed failure to induce immunological tolerance by such technics.

Those rats which did not succumb to progressive tumor growth following inoculation of C3Hf leukemia cells have remained apparently heathy and are kept under observation for the possible development of 'late lukemia'—leukemia induced in rats by a mouse leukemia virus, as reported by KIRSTEN *et al.*[7,34] in the heterologous transplantation of spontaneous AKR leukemia of proven viral etiology. Attempts to transmit the C3Hf leukemia into isologous newborn mice by cell-free filtrates prepared by the method of GROSS[35] have been thus far unsuccessful after an observation period of 15 months.

Aside from hepatic involvement by C3Hf heterotransplants, marked fatty change in the centrolobular liver cells was consistently evident. SIEGLER and KOPROWSKA[36] also observed a similar fatty change in the cells of the peripheral portions of liver lobules following homologous transplantation of a C3H ascites tumor, and ascrided the phenomenon to the altered metabolism associated with mobilization of the subcutaneous depot fat in the tumor-bearing animals.

SUMMARY

Spontaneous C3Hf lymphatic leukemia maintained in an ascites form was transplanted into newborn Wistar rats less than 24 hours old. Single subcutaneous or intraperitoneal inoculation of the neoplastic cells resulted in progressive tumor growth fatal to the heterologous hosts. Limited serial passage through newborn rats was performed.

The intraperitoneally heterografted leukemia grew as massive lymphosarcoma predominantly in the adipose and connective tissue compartments with invasion of the neighboring organs but without leukemic manifestations. The characteristic behavior and histopathologic features of the transplanted disease are presented in comparison with the results of similar experiments reported in the literature.

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EXPLANATION OF PLATES

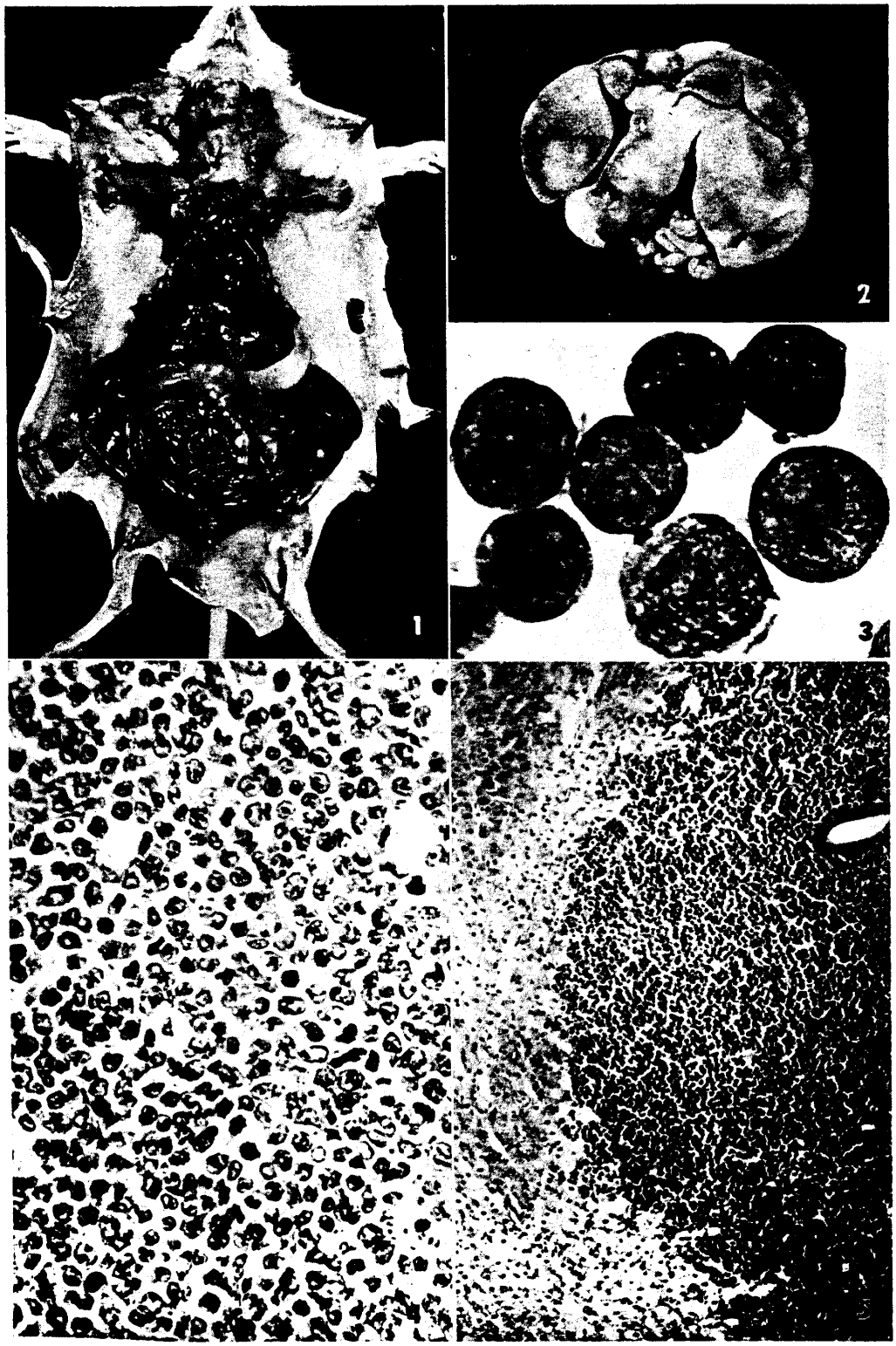
Fig. 1. Wistar rat which died from a massive abdominal tumor 20 days after intraperitoneal inoculation of C3Hf ascites leukemia cells at birth.

Fig. 2. Cross-section of the abdomen of a rat bearing transplanted C3Hf leukemia. Note tumorous, solid growths surrounding the kidneys and impinging upon the intestinal loops.

Fig. 3. Cell suspension smear of the rat tumor. × 1000.

Fig. 4. Histologic appearance of the rat tumor. Note numerous mitotic figures. × 400.

Fig. 5. Invasion of rat liver by C3Hf leukemia cells. Note a remaining bile duct. × 100.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EXPLANATION OF PLATES

Fig. 6. Centrolobular fatty metamorphosis of the uninvolved portion of tumor-bearing rat liver. × 100.

Fig. 7. Invasion of the wall of rat stomach by C3Hf leukemia cells. × 100.

Fig. 8. Invasion of rat stomach by C3Hf leukemia cells. × 100.

Fig. 9. Subcapsular and perivascular invasion of rat kidney by C3Hf leukemia cells. × 100.