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ASSOCIATION EURATOM - T.N.O.
(European Atomic Energy Community and Stichting voor
Toegepast Natuurwetenschappelijk Onderzoek

BONE MARROW TRANSPLANTATION IN THE RHESUS MONKEY

by

L.M. van PUTTEN

1964

Work performed under the Euratom contract No. 004-59-12 BIAN

**Reprinted from the
Proceedings of the "International Symposium on Bone Marrow
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INTERNATIONAL SYMPOSIUM ON BONE MARROW

THERAPY AND CHEMICAL PROTECTION

IN IRRADIATED PRIMATES 1962

BONE MARROW TRANSPLANTATION IN THE RHESUS MONKEY

Progress Report

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Studies on the protection of rhesus monkeys after lethal irradiation doses with bone marrow suspensions have been reported from our institute (Crouch et al., 1961; de Vries et al., 1961). Summarizing these data it may be concluded that:

1) With fresh autologous marrow, protection against doses up to about 1000 r is routinely obtained with 1.2×10^8 cells per kg body-weight or more.

2) With homologous marrow, takes of donor material could be demonstrated after $2.5\text{-}3 \times 10^8$ cells per kg upward; however, long survival was never seen (max. 9 weeks). The animals died from "secondary disease" with the following clinical symptoms: anorexia, diarrhea, skin lesions, jaundice, infections (the post mortem findings were summarized by de Vries in the previous paper).

3) Secondary disease was seen only when a take of the donor cells occurred. It occurred usually very early after bone marrow transplantation; there was some variation in the severity of the disease but it was generally much more severe than usually seen in rodents and it proved uniformly lethal.

* This work was performed under contract with Euratom (European Atomic Energy Community) 51-53 rue Belliard, Brussels, Belgium.

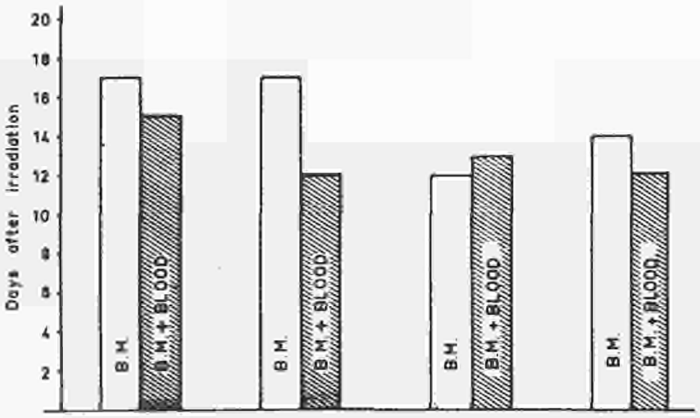
The present report is mainly concerned with studies on the cause of the severity of this secondary disease and with means to prevent it.

Since most of the earlier studies were performed with cells obtained by puncture and aspiration of bone marrow from living donors, the cell suspensions were inevitably contaminated with relatively large quantities of blood.

In rodents it has been established that admixture of the foreign marrow suspensions with donor blood markedly enhances secondary disease or even has an acute killing effect on the 6th or 7th day after administration (Cole and Garver, 1961; Goodman and Congdon, 1961).

To establish whether in monkeys a similar effect of peripheral blood was responsible for the severe secondary disease, the following experiments were performed.

Figure 1



Survival times of X-irradiated monkeys (650 r) after treatment with bone marrow alone or bone marrow + 30 ml blood.

Donor monkeys were bled to death under anaesthesia and their bone marrow was collected. One part of the suspensions was mixed with 30 ml of blood and administered to one lethally irradiated monkey, another part was mixed with Tyrode's solution and injected into another similarly irradiated monkey. No marked differences in survival were seen. Autopsy showed severe secondary disease in all animals (fig. 1).

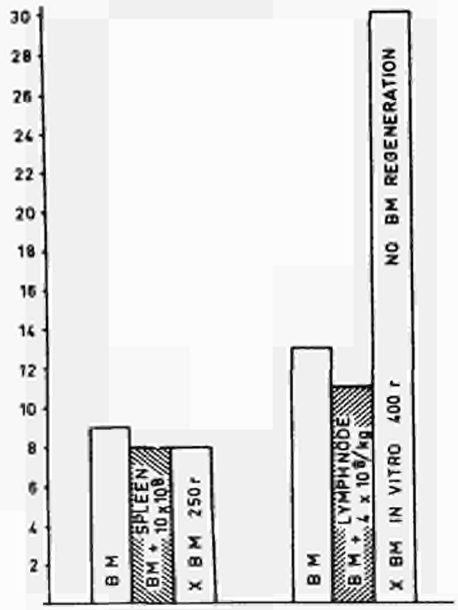
Another experiment was designed to elucidate whether the acute killing effect as seen in rodents after injection of peripheral blood, or large numbers of lymph node or spleen cells, could be reproduced in the monkeys.

Large numbers of immunologically competent cells (10×10^8 spleen cells or 4×10^8 lymph node cells per kg) were injected in addition to 5×10^8 bone marrow cells per kg body-weight of the X-irradiated recipient. This caused neither a markedly shorter survival time (fig. 2) nor any special clinical symptoms or autopsy findings in these monkeys in comparison with the controls, that received bone marrow alone from the same suspensions.

This result may be explained in two ways. Either lymphoid cells have nothing to do with secondary disease and with the mortality of these animals - a thesis which is contradicted by the pathological studies of de Vries (see previous paper) - or the bone marrow suspensions alone contain already a maximally effective number of immunologically competent cells. This latter supposition would be in agreement with the finding that lymph node repopulation usually seems to precede bone marrow repopulation after administration of bone marrow.

The next step in our studies is a logical consequence of the finding of an excessive immunological activity of the bone marrow suspensions: Attempts were made to reduce selectively the number of immunologically active cells

Figure 2



Survival times of X-irradiated monkeys (850 r) after treatment with bone marrow (5×10^8/kg) or with bone marrow (5×10^8/kg) + lymphoid cells from lymph nodes or spleen or with X-irradiated bone marrow (20×10^8/kg).

in the suspensions by various methods.

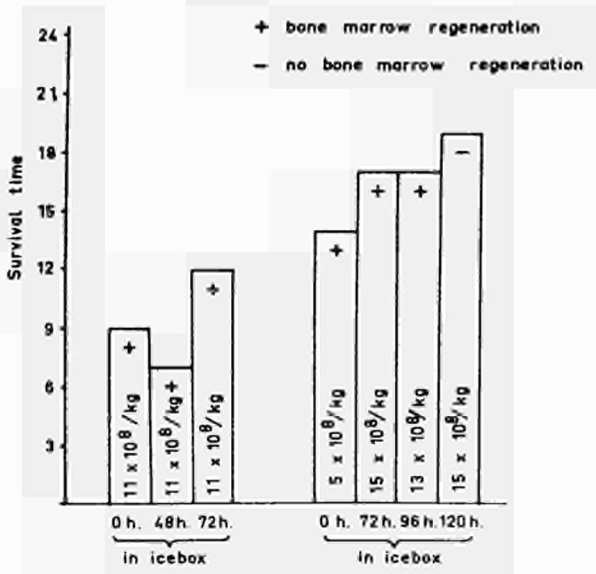
a) Irradiation might favourably influence the relative immunological and hemopoietic potencies of the cell suspensions. Irradiation was tested with an increased number of cells administered, to compensate for a partial loss of hemopoietic cells.

A fourfold increase in cell number combined with in vitro irradiation of the suspension with 250 r of X-rays did not change survival time or severity of secondary disease. A similar experiment with a fourfold increase in cell number and 400 r of X-rays resulted in a longer survival time with evidence

of temporary and incomplete regeneration of bone marrow (fig. 2) but with at the same time definite evidence of a minor degree of secondary disease.

b) Another method was found to be much more successful in mice in preventing homograft reactivity from mouse spleen suspensions without equivalent loss of radiation protection potency (van Bekkum, 1962). This method consists simply of storage at + 4°C for 3 or 4 days. The results in monkeys are shown in figure 3. It is evident again that the longest survival time is obtained where no bone marrow regeneration occurs. There

Figure 3

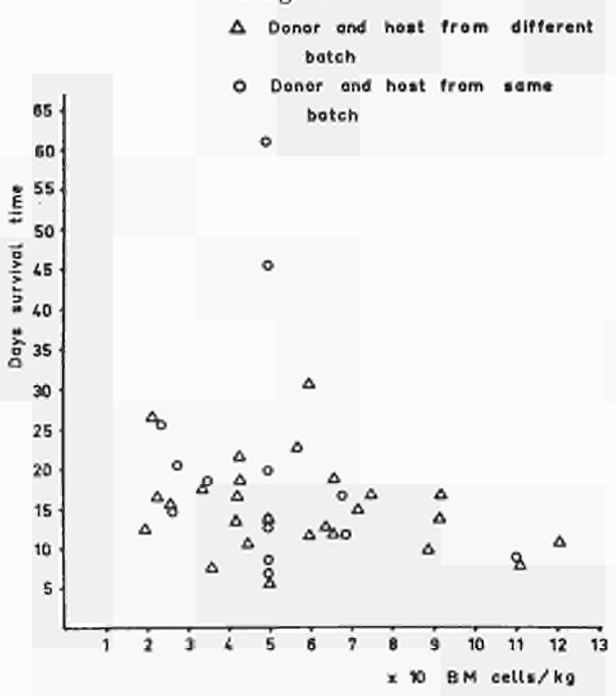


Survival times of monkeys treated 24 hours after 850 r total body irradiation with bone marrow stored at + 4°C for various periods.

may be a slight effect of storage but this has certainly no clinical importance. These observations suggest that methods which are effective in

decreasing the secondary disease in rodents are not necessarily effective in the monkey. This could be due to resistance of monkey lymphoid cells but it could also be due to an excess of immunological potency of the bone marrow suspension. If the latter is true it is rather surprising that we ever observed survival times of over 30 days and it might be worth-while to reconsider these results. If these long survivors were not obtained by low immunological activity of the suspension, they could perhaps be a result of variations in the degree of "compatibility" between host and donor.

Figure 4



Survival times of monkeys after irradiation and homologous transplantation plotted against bone marrow cell dose. Donor-host combinations from possibly related monkeys, arriving in the same batch are presented as circles; the others as triangles.

With this in mind we have tabulated all our data in a search for compatible and incompatible donor-host combinations. Most of the studies have been done with monkeys which arrived in this country in different transport batches. Only in a few instances donor and host were selected from the same batch. Among these instances were our two longest survivors but the total average of this group is not significantly better (fig. 4). We are thus unable to conclude whether selection of "compatible" or related monkeys may improve the results.

Finally we have started work with fetal donor material. Satisfactory suspensions from fetal monkey liver and spleen could be obtained from fetuses of an estimated age of 100 days (after the last menstrual bleeding). The number of cells obtainable from one fetus was at best only slightly more than the minimal number of adult homologous cells (8×10^8 for a 3 kg recipient) necessary to obtain a "take". Nevertheless in two trials no effect of these numbers of hemopoietic cells was observed in irradiated recipients and several attempts with lower numbers of cells have also been unsuccessful.

A mixture of suspensions from two fetal donors was tried once, supplying 7.2×10^8 cells per kg recipient. This produced temporary incomplete bone marrow recovery and incomplete lymph node recovery and survival for 21 days, histologically there was no evidence of secondary disease.

It seems that just as in mice (Crouch, 1960) more donor cells may be needed from fetal than from adult sources to produce a similar degree of hemopoietic recovery. For monkeys this seems to imply that more than one fetal donor is certainly needed and we plan to test large numbers of donors. We do not know what are the effects of administering suspensions made up from a number of donors. For practical application it will almost certainly be necessary to have a dependable freezing method, but we believe the treatment should first be worked out using pooled fetal cell suspensions.

As regards the freezing method, preliminary data have indicated that the storage of monkey bone marrow suspensions causes in our hands a much greater loss of protection ability than when the same storage techniques were applied to mouse bone marrow.

Recent experiments in our institute demonstrated that the standard slow freezing in 30% glycerol-tyrode with rapid thawing and Sloviter procedure gives for mice about 60% preservation of the fresh protective effect. For monkeys the preservation percentage seems in preliminary studies to be below 10 and we have as yet not been able to protect a single animal with autologous marrow after freeze-storage.

In summary we feel that notwithstanding a large number of negative results, the subject has not been covered exhaustively and there is still a lot of work to go through before all the possibilities of preventing secondary mortality have been explored.

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DISCUSSION

KURNICK When exactly did Dr. van Putten determine the viability of his foetal cells? In our limited experience with human foetal cells we found that within an hour the viability, as measured by trypan blue exclusion, had fallen to almost zero, both for foetal spleen and bone marrow. Concerning the use of frozen stored bone marrow we found that the viability is much improved by avoiding dilution by the Sloviter method (H. A. Sloviter. Am. J. Med. Sci., 231, 437, 1956). We found no harm in injecting it in the presence of glycerol without filtration and without removing fat. Slow thawing at zero degrees C in air seemed to have an advantage over fast thawing.

VAN PUTTEN As to the first question, we do find a reasonable viability of the monkey foetal material using the eosin-test. Some of the foetuses are obtained from an institute where great numbers of monkeys are sacrificed for polio investigations. If a pregnancy is found, the foetus is put on ice and received by us within 3 hours. Roughly 7% of the cells of our foetal marrow suspensions are dead. Foetal livers of a suitable age yield suspensions with up to 60% non-viable cells, the dead cells being mainly parenchymal, non-hemopoietic cells. I must admit however that our only case of repopulation by foetal material, was when the suspensions were rapidly obtained from 2 foetuses who were delivered by cesarian section in our laboratory and were injected soon afterwards. In answer to the second question I would like to mention that in a statistical study in mice, undiluted glycerol-stored suspensions are much less effective in saving irradiated animals than the same suspensions if diluted by the Sloviter method. However if dimethyl sulfoxyde is used in a 15% concentration for storage, the undiluted suspension is very effective but the Sloviter dilution, in this case, completely abolishes the protective effect.

KAY would like to confirm the difficulties with eosin, when testing foetal

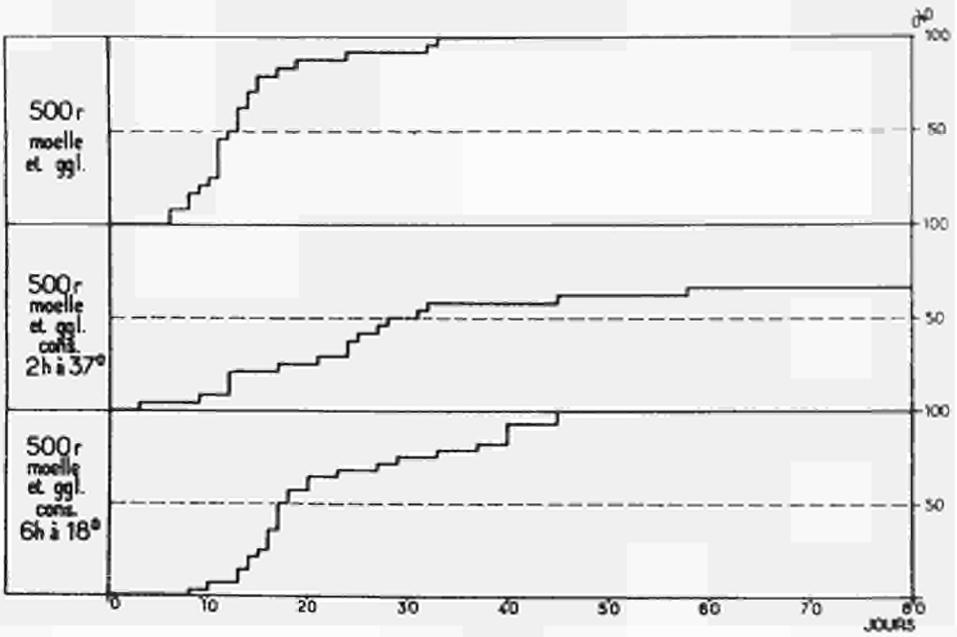
material, since all parenchymal cells take up the dye. He uses acridine orange which produces an orange fluorescence in viable cells. This correlates well with the biological activity of cell suspensions after freezing with dimethyl sulfoxide (which is preferred to freezing with glycerol).

VAN PUTTEN Could a rough estimate be given of the percentage viable cells recovered after freezing?

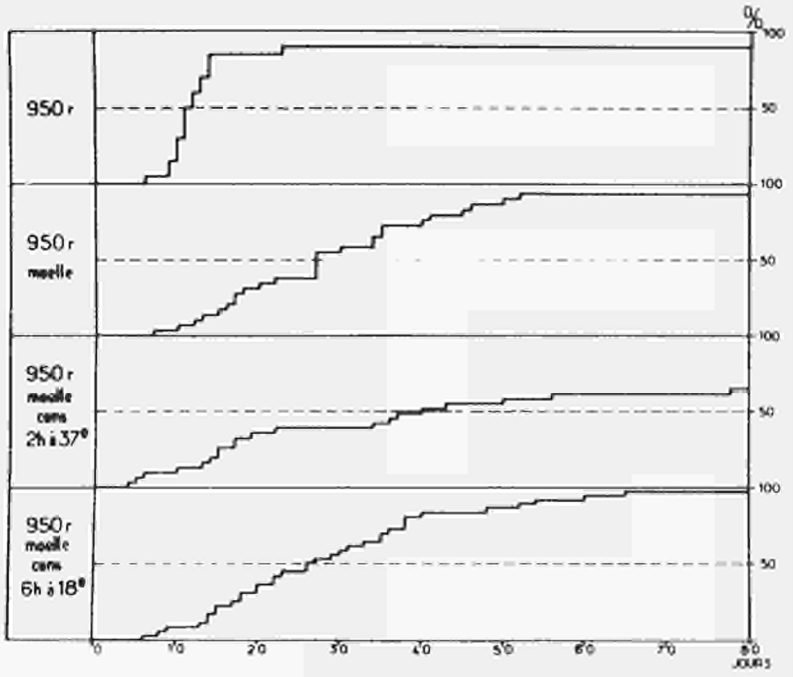
KAY Yes, with the optimum concentration of dimethyl sulfoxide (12.5%) one gets about 40% of the original number of viable cells. With human HeLa or lung culture cells one can get 80% recovery by a Puck plating technique so that the results are not only species-specific but also depending on the cell type.

VOS As has been mentioned by Dr. van Putten, attempts have been made to eliminate selectively lymphoid cells from bone marrow in order to reduce secondary disease. One of the methods is based on X-irradiation, but when Smith and I tried to estimate the radiosensitivity of lymph node cells and marrow, the LD_{37} was not significantly different for the two types of cells in mice. There seems to be little hope to kill either of these two cell types selectively.

MATHÉ would like to mention another way of conservation which seems to affect the immunologically competent cells somewhat more than the myeloid cells. Conservation of cells for 2 hours at 37°C or 6 hours at 18° reduces the number of eosin-resistant cells to about 50%. The three tests used are depicted in the slides. The top row shows the mortality when we inject C57BL marrow and lymphoid cells into sublethally irradiated F_1 (C57 x DBA2) and look for a killing effect and one can see that only the conservation for 2 hours at 37° (second row) decreases mortality significantly (slide 1). In the second experiment only homologous marrow was used and one can see that conservation for 2 hours at 37°C decreases lethality significantly. Though

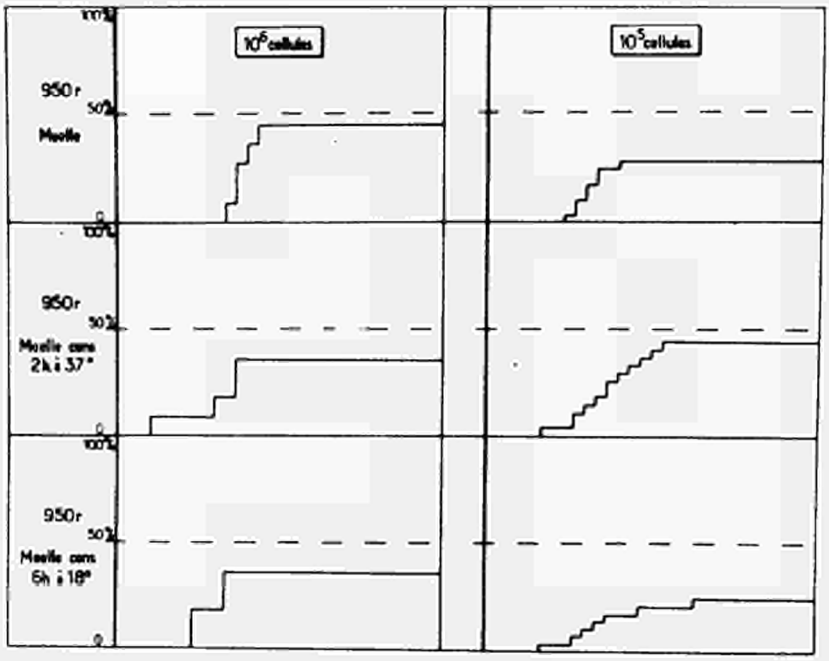


Slide 1



Slide 2

myeloid restoration is not decreased, storage for 6 hours at 18° does not work (slide 2). Then, in the third experiment where restoration was obtained by minimal numbers of isologous marrow cells (10^5 and 10^6) no significant differences are observed between the three groups (slide 3). But



Slide 3

one loses 60% of the cells and to inject the same number of viable cells one would have to start with twice that number of cells. If we want to apply this to humans we would need more cells than can be obtained from one individual so we expect to use several donors per recipient to test this.

VAN BEKKUM Unless minimal effective numbers of cells were used in the first two tests your results do not prove a selective effect on either of the two cell types. From my experiments there seems to be a slight selective effect at last and the only real proof for this is the following. In certain mouse host-donor combinations, parent spleen suspensions will not provide

EFFECT OF STORAGE AT 4°C ON THE KILLING EFFECT OF LYMPHOID CELLS

donor cells CBA ♂		irradiated recipients F_1 ♀♀	
		percentage mortality	
5×10^6 marrow + 2×10^5 lymph node cells		30 days	60 days
fresh	(14%)*	90	90
3 days	(27%)	25	100
4 days	(33%)	25	40
5 days	(42%)	100	-
20×10^6 spleen cells			
fresh	(34%)	100	-
3 days	(40%)	45	56
4 days	(71%)	56	66

* percentage of eosin positive cells in suspension

Slide 4

protection to irradiated F_1 hybrids regardless of the number of cells injected, the mice die either from direct killing or from bone marrow aplasia. If however the suspension is stored in the refrigerator for a few days before injection we do get a certain percentage of survivors, though the protective effect is rather small (see slide 4). Therefore, I would like to ask you whether you have done cell number titriations in your tests and whether you found the effect when a minimal effective number of cells was used.

MATHÉ In the case of isologous restoration (slide 3) we worked with 10^5 and 10^6 cells and found the same protection in both. We do not have 100% protection and we are not down to the minimum level. But it seems that we can decrease the secondary syndrome in practice by this method. That is, if we can get more donor cells.

VAN BEKKUM agrees that it is very difficult to prove a selective effect on immunologically active cells and that one must keep in mind that any decrease of the number of cells of a suspension capable of causing a graft-

versus-host reaction, will indeed reduce the chances for this graft-versus-host reaction to become manifest.

AMBRUS Did Dr. Mathé actually propose to give irradiated patients marrow from several donors at a time? Would this not complicate matters by adding a multiplicity of graft-versus-graft reactions to the already existing immunological reactions of graft-versus-host and vice versa?

MATHÉ With this in mind we have restored irradiated mice with marrow from 4 different strains, including C57 marrow causing a high percentage of secondary disease and DBA2 marrow which rarely provokes a secondary syndrome. When these 4 marrow suspensions are mixed we observe less secondary syndromes than when the strain with maximal anti-host activity (C57) is used alone.

AMBRUS wonders how the experiments are actually set up since one must consider the possibility that putting inother grafts next to the C57 marrow, which is so damaging to the host, the additional grafts may simply destroy part of the primary C57 marrow graft. One would only get a seemingly better result.

MATHÉ The experiment is now in its 50th day and apparently less secondary disease is seen than when only C57 cells were used.

COHEN would like some more comments on the use of marrow pools. Though the problem of graft-versus-host reactions as mentioned by Dr. Ambrus must be kept in mind, Lengerova of the Immunological Institute at Prague, gets quite favourable results by using large pools of some 40 types of cells. This might be due to a "self-clearance effect" of lymphoid cells. Something like a "clonal selection mechanism" might be operative, by which the antigenically remote cells perish while those antigenically acceptable to the host might be saved. Maybe it would be useful to continue the kind of work Lengerova has been doing with pooled cells, in the hope of

saving selectively those immunologically competent cells that would be antigenically most related and most acceptable to the host.

CROUCH Had anyone in the group ever done this in monkeys?

LOEB mentions the use of 3 donors for the treatment of patients. The different marrows were not injected simultaneously but successively within a few days. Death in these children occurred 28 to 30 days after total body irradiation. Secondary disease may have played a role in the death of these children: the lymphoid tissue was definitely depleted in 4 out of 5 at autopsy. The marrow of both parents and of other close relatives was used in these cases.