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ANNUAL SUMMARY REPORT

CONTROL OF SECONDARY SYNDROME FOLLOWING WHOLE BODY IRRADIATION TREATMENT WITH BONE MARROW TRANSPLANTS

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Professor G. MATHE
Director of "Insitut de
Cancérologie et d'Immuno-
génétique. Hôpital Paul-
Brousse. 94 - VILLEJUIF,
FRANCE.

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ABSTRACT

1) A test is described that enables the action of antimitotics and other agents on immunologically competent cells "in vitro" to be studied. This test is rapid, and the results reproducible, it consists of incubating mouse spleen cells from animals immunized against sheep erythrocytes with the compound to be tested. The drugs are used in the concentrations that are equivalent to those that 1) produce an LD 50 cytostatic effect "in vitro"" ; 2) cause 50 % of the cells to be permeable to trypan blue after they have been in contact with the drug in Tyrode solution for one hour. This test has been applied to many different drugs, from it we have now derived a test for the routine study of immuno-suppressive drugs.

2) We have studied the nature of the immune reactions of a secondary syndrome that follows the transfusion of allogeneic leucocytes.

3) We have continued the study of the different conditions under which the secondary syndrome can be prevented by the incubation of the allogeneic haematopoietic cells at 37°C.

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5) We have undertaken a study of a possible antigeneic competition, by giving an animal, at the same time, antigeneic stimuli that differ quantitatively or qualitatively.

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Le traitement symptomatique de l'agranulocytose par les transfusions de globules blancs. SCHWARZENBERG L., MATHE G., AMIEL J.L., CATTAN A., SCHNEIDER M. et SCHLUMBERGER J.R. La Presse Médicale, 1966, 74, 1057.

Conditioning of immunologically competent cells by incubation at 37°C. MATHE G., AMIEL J.L., SCHWARZENBERG L., DORE J.F., GOLDSTEIN P., SEKIGUCHI M. and BECHET J.M. 7th International Conference of Transplantation, New-York, 1966.

BODY OF REPORT

The work carried out under this contract is concerned with the possibilities of preventing or controlling the immune reactions that follow the grafting of immunologically competent allogeneic cells into a recipient. The recipient may have been or not specifically prepared before grafting.

I. STUDY OF THE EFFECTS OF DIFFERENT CHEMICAL COMPOUNDS ON IMMUNOLOGICALLY COMPETENT CELLS.

We have continued the study of different rapid test systems to examine the effects of drugs on immunologically competent cells in vitro. The purpose is to be able to evaluate the importance of various types of chemical compounds, either with or without anti-mitotic activity, in attentuating the immune reactivity of the cells prior to their transfusion. We have now developed a test system that is in routine use in our laboratory. The principle of this test is as follows : Spleen cells from mice immunized with sheep red cells, are incubated at 37°C for one hour with the drug to be tested. The effects are assessed by comparing the number of plaques of hemolysis formed by the cells when plated on sheep red cell gel plates, to the number formed by cells incubated without the drug. F1 (CBA × C57Br) mice are given a single intraperitoneal injection of 0.5 ml of a 10 % solution containing 10^9 red cells, 4 days later the animals are killed and the spleens removed. The spleen cells are suspended The spleen cells are suspended in TC 199 at a concentration of 5×10^7/ml. We distribute 0.5 ml of the cell solution, the control tubes contained 0.5 ml cell suspension + 0.5 ml Hank's solution or 0.5 ml of the solvent used for the drug if it was not water soluble. The tubes are incubated for one hour and then the viability is assessed by the trypan blue uptake test.

The suspensions are then adjusted to contain 10^7 viable cells/ml. 0.1 ml is taken and mixed with 2 ml Hank's solution containing 0.6 % Agar and 0.1 ml sheep red cells at 37°C. The mixture was poured into a Petri dish containing a layer of 10 ml PBS containing 1.2 % agar. We have studied twenty substances by this method, which has clearly

illustrated the remarkable effect of 6-mercaptopurine on the immunologically competent cells, the action of weak, non cytotoxic doses of delta-cortisone, the apparently selective effect of methyl-hydrazine chlorhydrate.

Several critical questions need to be considered.

1° The permeability to trypan blue gives a good idea as to overall cytotoxicity of the drug, or a specific action on the cellular permeability may be possible.

2° The disappearance or reduction of the plaques of lysis could be due to destruction or inhibition of antibody forming cells, or to a liberation during the incubation of preformed intracellular antibodies. We now intend to combine isotopic marking techniques and autoradiography with this method and follow the metabolism of the cells submitted to these treatments.

The method has a good reproducibility and a good correlation was found between the results obtained by this method and those previously obtained by our more complex immuno-suppressive tests "in vivo", on substances such as 6-mercaptopurine and methylhydrazine. We believe that this test will be very useful for the study of drugs on the immunologically competent cells.

II. STUDY OF THE SECONDARY SYNDROME FOLLOWING THE TRANSFUSIONS OF LEUCOCYTES IN PATIENTS WITH BONE MARROW APLASIA.

We have treated patients with malignant blood disorders in an aplasic state, that had either arisen spontaneously or as the outcome of intensive chemotherapy or extensive radiotherapy, by leucocyte transfusions. The leukocytes were taken from either patients with chronic myeloid leukemia or from a pool of normal donors. This treatment has shown to induce a syndrome, closely resembling the secondary syndrome which complicates the grafting of allogeneic bone marrow. This effect is certainly dependent on a graft versus host reaction. The secondary syndrome may be seen after transfusion of fresh leucocyte as well as when leucocytes stored at - 70°C are used, though it is clearly less marked in the latter case.

Previously we have been able to show in the mice that incubation of the cells for 37° for 2 hours considerably affects the immunologically competent cells and diminishes the severity of the secondary syndrome. In an attempt to reduce the intensity of the immunologic reaction after transfusion of allogeneic leucocytes, we have undertaken a clinical trial using cells that have been incubated for 2 hours at 37°C. The first results observed using this method have demonstrated that the incubation does not diminish the effectiveness of injections of leucocytes in combating infections, and does not diminish the transitory myeloid graft. Though, this pre-treatment of the cells considerably reduces the risks of the secondary syndrome. Since putting this method into practice we have never observed the secondary syndrome following the injection of circulating allogeneic leucocytes, which could not be controlled easily by symptomatic treatment.

III. We have followed up our trials of allogeneic bone marrow after total body irradiation at 800 rads in patients with acute leukaemia resistant to all other therapy. The treatment now takes place in a germ free area (G.F.A.) in the Institut de Cancérologie et d'Immunogénétique. The selection of a recipient who has never been previously transfused and using several donors, to obtain a minimum of 5×10^{10} cells has enabled us to obtain in our latest 7 patients, 6 successful myeloid restorations, 5 of whom died from an acute or subacute secondary syndrome which we described in our previous report. Our attempts at present to attenuate the secondary syndrome are as follows : 1) by treatment with methotrexate immediately following the irradiation and the graft ; 2) by treatment with a single dose of cyclophosphamide when the earliest signs of a secondary syndrome during the second week after the irradiation and the graft. It would appear in man the best chance of controlling the secondary syndrome is to eliminate those donors against whom the recipient shows a marked antigeneic reaction, and whose cells, when injected, induce a violent graft versus host reaction. We have tried to eliminate such donors by 1) study of leucocyte phenotypes, 2) by testing of the reaction of 2 populations of allogeneic lym-

phocytes coming from the donors and the recipient and injected into the skin of a lethally irradiated hamster.

IV. CONDITIONING OF IMMUNOLOGICALLY COMPETENT CELLS BY INCUBATION AT 37°C.

The success of incompatible grafts requires a certain control of immune reactivity, in the case of non-haemopoietic grafts of the host's immunologically competent cells (ICC) and in the case of haemopoietic grafts, of both the host's and the grafted ICC.

A systematic study of various methods of conditionning ICC has shown their extreme sensitivity to incubation at 37°C.

A) Analysis of the effects of incubating immunologically competent cells at 37°C.

Let us first consider various experimental findings which allow us to state that ICC are sensitive to being kept "in vitro" at this temperature.

Jerne's method shows that, if mouse splenic cells immunized against sheep red cells are incubated for 2 hours at 37°C in Tyrode solution, Hanks solution or autologous serum, regardless of whether they are agitated or not, a 73 p. 100 reduction in the number of cells able to form haemolytic plaques occurs. A reduction of 56 p. 100 occurs after one hour. The size of the plaques obtained is equally and significally reduced if the cells have been incubated.

The early normal lymphocyte transfer reaction of two populations of lymphocytes injected intradermally in hamsters given 950 rads of irradiation 6 hours before, the reaction being measured each day by infra-red thermometry is depressed if the cells have been previously incubated for 2 hours at 37°C. This applies to both mouse cells and human cells.

A study of the graft versus host reaction in mice shows that if lymph node cells of C57Bl/6 animals are incubated in Tyrode solution for two hours before being injected intravenously into F1(C57Bl/6 × DBA/2) hosts which had received a total body irradiation of 500 rads, it requires more than 4.10^7 cells to produce 50 p. 100 mortality, as compared to 10^6 non-incubated cells. Similar

results are obtained if the cells are suspended in autologous serum.

In conclusion, whether the ICC are mouse or human cells, whether they arise from donors either previously immunized by antigen or not, whether the medium in which they are incubated in Tyrode, Hank's or autologous serum, or whether they are agitated or not while being incubated at 37°C, their functional capacity to react immunologically is constantly impaired after incubation at 37°C for 2 hours.

Mechanism

The responsible mechanism has as yet not been elucidated. We know however that no gross alteration in the cells occurs, for example due to simple pH modification.

Other types of cells incubated under the same conditions are apparently unharmed.

Thus, the restorative value of isogeneic bone marrow grafts given to 100 p. 100 lethally irradiated animals is not appreciably altered when the marrow has first been incubated for 2 hours at 37°C.

This sesnsitivity therefore seems to be characteristic of ICC.

Finally one has consider whether it influences lymphocytes, cells which produce or are destined to produce antibody, or whether it affects cells which are believed to intervene or to be able to intervene directly or indirectly in the production of antibody, ie. macrophages and lymphoblasts. This latter group, the precursors of lymphocytes, does not seem to be any more affected than the stem cells of myeloid tissue ; thus lymphocyte restoration after isogeneic bone marrow grafting of lethally irradiated animals does not differ, regardless of whether the marrow was previously incubated or not. The macrophages have been studied by marking them with warbon. They do not appear morphologically to be grossly affected. Only some lymphocytes show morphological anoamlities.

We are attempted to analyse the mechanism wereby incubating lymphocytes at 37°C affects antibody formation.

Keeping these cells in this fashion does not seem to affect their ability to proliferate. In the graft versus host reaction in mice, the increase in weight of the host's lymphoid organs, which we have shown in this experiment to be essentially linked to the proliferation of the grafted cells, is identical to the control group and the histology of the lymphoid tissue shows the same proliferation of these cells. Human lymphocytes taken from theperipheral blood and brought into contact with phytohemagglutinir (PHA) show the same mitotic index regardless of whether they were previously incubated at 37°C for 2 hours or not.

RNA synthesis is under investigation, but it does seem as if total cellular RNA synthesis is unaffected. The number of hyper-basophilic cells and the intensity of their basophilia is not diminished in the graft versus host reaction in mice. Total RNA synthesis after contact with phytohemagglutinin, studied by incorporation of tritiated uridine (incubation for 1 hour with 2,5 c/ml) is not reduced significantly. Total protein synthesis after this contact, studied by incorporation of tritiated L phenylalanine (incubation for 2 hours 15 minutes with 100 c/ml after a 3 days contact with PHA) is not reduced significally eitheir.

The underlying mechanism of this process seems then to be specifically related to antibody production, but not demonstrable by gross investigation of the proliferation power of the ICC and of total RNA and protein synthesis.

The present interest in this phenomenon is its simplicity of usage in the control of the host versus graft reaction as well as that of the graft versus host reaction.

B) Conditioning of recipients for allogeneic grafting.

F1 (DBA/2 x C57Bl/6) mice irradiated with 800 rads can be restored by 10^6 isogeneic bone marrow cells, fresh or incubated for 2 hours at 37°C. Mice restored by cells which have been incubated show a higher degree of tolerance towards an allogeneic F1 (DBA/2 x Balb/c) skin graft than mice restored by fresh cells (21 out of 33 animals have a tolerance longer than 25 days in the 1st group ; only 8 out of 32 show a similar tolerance in the 2nd group, while there were none in the control group). Myeloid

restoration is not affected by incubation of the bone marrow.

At the present time we are using this method to promote the induction of immunologic tolerance to allogeneic grafts in larger animals (rabbits, swine, monkeys) using autologous bone marrow incubated at 37°C while the animals is irradiated.

C) Control of the secondary syndrome complicating allogeneic bone marrow grafting.

The main immediate application, which has already been introduced to the clinical situation, is the control of the secondary syndrome complicating allogeneic hemopoietic tissue grafting.

If the bone marrow of C57Bl/6 mice is incubated at 37°C for 2 hours befor being transfused into F1 (AkR x C3H) mice irradiated with 950 rads, the incidence of secondary syndrome is notably reduced compared with the control group.

Similarly if bone marrow is incubated at 37°C for 2 hours, the incidence and intensity of the runting syndrome, induced by intravenous marrow grafting at birth is considerably reduced.

Finally, in the course of the clinical therapy of extreme leukopenia we were able to demonstrate that the transfusion of white cells (obtained from donors suffering from chronic myeloid leukaemia), may by followed not only by a take of the graft (as shown by examination of the leucocyte sex appendages and by chromosomal analysis), but also by a secondary syndrome clinically and histologically the syndrome which complicates allogeneic bone marrow grafting. Again, keeping the leucocytes at 37°C for 2 hours seems to reduce the incidence and, more importantly, the seriousness of this complication ; it should be noted however that the number of cases is as yet small and the results obtained therefore not statistically.

Obviously, this method of preventing secondary syndrome should be considered in allogeneic bone marrow grafting in Man when an extremely severe graft versus host reaction is feared or when therapeutically the graft versus host reaction is unwanted all together.

In summary, incubation for 2 hours at 37°C of immunologically competent cells reduces their functionnal capacity to react immunologically while it does not alter the restoration power of the myelopoietic and lymphopoietic stem cells. This phenomenon can be used in the control of the host versus graft reaction as well as the control of the graft versus host reaction.

V. STUDIES OF THE USE OF SEVERAL SIMULTANEOUS ANTIGENIC STIMULI.

We have conducted research in to whether it is possible to diminish the response to an antigenic stimulus by giving the animal another antigenic stimulus that is qualitatively different and quantitatively greater.

1) Swiss mice were immunized with human albumin in 4 intraperitoneal injections during to weeks. These were given either alone or each injection accompanied by an intravenous injection of sheep red cells, 10^4 cells in one group, 10^6 cells in a second group, 10^9 in a third group. The immunization against the human albumin was studied, on the 10th day after the last injection, using the clearance of I^{131} labelled antigen. No difference between the controls and those groups that received simultaneous injections of human albumin and sheep red cells.

2) We have used an experimental design which iscomparable to the reaction of the grafts, C57Bl/6(H-2^b) mice were grafted with the skin of C3H(H-2^k) mice. In one group this graft was applied alone, in other groups the animals were given, at the same time, an intraperitoneal injection of L1210(H-2d) leukemia cells, 10^5, 10^6, 10^7 cells to different groups of mice.

No statistical difference was found between the tolerance of the C3H skin grafts in any of the treated groups and the controls.

These experiments appear to show that it is not possible to diminish the immune response to an antigenic stimulus by submitting the animal simultaneously to another qualitatively different antigenic stimulus, whatever the "volume" of this second antigenic stimulus.