REQUIREMENT OF GLYCINE OR ITS POLYMERS IN THE STIMULATION OF HUMAN PERIPHERAL LYMPHOCYTES BY PHYTOHEMAGGLUTININ

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1. Introduction

In the process of mitogenic stimulation of lymphocytes a general increase of metabolism occurs, leading to the mitosis of the cells [1]. In order to obtain an optimal stimulation it is therefore necessary to provide the cells with a balanced nutritional medium and serum. The role of the serum is not well understood. It is, however, well established that in the early phase of stimulation the dialysable part of the serum (diffusate) could effectively replace the whole (intact) serum [2].

In this work we present evidence that glycine, free or bound in peptides, could to a large extent replace the serum diffusate in lymphocyte cultures, stimulated by phytohemagglutinin. Furthermore, we wanted to find out the largest glycine peptide remaining still active in this system.

2. Materials and methods

Human peripheral lymphocytes were separated from the blood of healthy donors. The lymphocyte rich layer, obtained by sedimentation of the cells, was separated and washed three times with Eagle's Minimal Essential Medium (MEM) (Difco). This medium was also used as culture medium with supplements of glutamine (0.32 mg/ml), 2-mercaptoethanol (2.5×10^{-5} M), penicillin (150 U/ml) and streptomycin (150 μg/ml). In experiment described in table 2, Dulbecco's medium containing glycine (30 μg/ml) was used instead of Eagle's MEM. The latter being free of glycine. In some cultures (as indicated) phytohemagglutinin P (Difco) (1 μl/culture) was present. We found this concentration to induce maximal stimulation under our experimental conditions. The cell suspensions, containing 0.5×10^6 nucleated cells/ml in a total volume of 2 ml, were incubated in Falcon tubes (No. 2027) for 66 h at 37°C in an atmosphere of 10% CO_2, 7% O_2 and 83% N_2. After 48 h 0.1 μCi of [2-^{14}C]thymidine (61 mCi/mmol) was added to each culture. Eighteen hours later the cells were filtered through a millipore membrane filter, then washed with 0.85% NaCl and 5% trichloroacetic acid. After the filters were dried, their radioactivity was counted in a scintillation liquid, composed per liter of 5 g PPO, 0.25 g POPOP, 333 ml Triton X-100 and 667 ml toluene. Each culture was carried out in duplicate.

3. Results

In order to obtain a stimulation of DNA synthesis by PHA in cultures containing Eagle's MEM, the presence of fetal calf serum in the medium was necessary (fig.1). However, if this serum was excessively dialysed, the activity was diminished and could be recovered by the addition of the diffusate, obtained by equilibrium dialysis of the serum against an equal volume of saline. The experiment shown in fig.1 also illustrates that an ammonium sulfate fraction (40–65%) prepared from fetal calf serum was similarly active as the latter, provided the diffusate was present in the culture. This indicates that all macromolecular components, necessary for a full activity of the system, were present in the 40–65% ammonium sulfate fraction of the fetal calf serum.

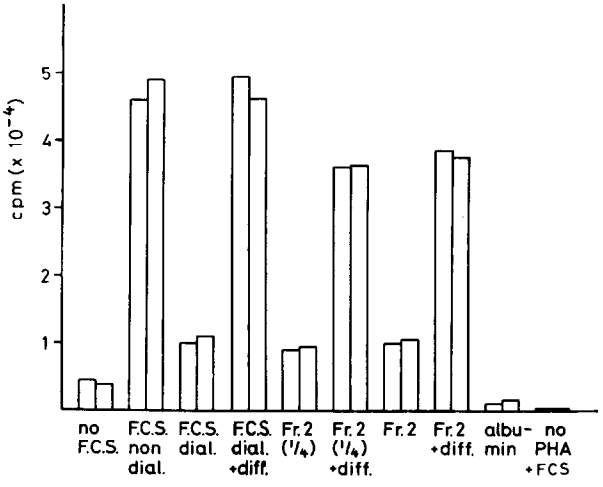

Fig.1. Effect of fetal calf serum on DNA synthesis of human blood lymphocytes stimulated by phytohemagglutinin. Human lymphocytes were cultured in Eagle's MEM with supplements (see Materials and methods). The dialysis of fetal calf serum (F.C.S.) was carried out at 4°C, for 48 h against an excess of 0.85% NaCl. The diffusate (diff.) was obtained by dialysis of a double concentration of F.C.S. against an equal volume of 0.85% NaCl for 20 h at 4°C. Fraction 2 (Fr. 2) represents a 40–65% ammonium sulfate fraction of the F.C.S. The final volume of this fraction was adjusted to be the same as that of the serum taken for fractionation. The concentration of the dialysed, nondialysed F.C.S., diff. and Fr. 2 was 5% (v/v) respectively. In two groups (as indicated) the concentration of Fr. 2 was 1.25% (v/v). With the exception of the last experimental group, phytohemagglutinin (PHA) was present in all cultures. The value obtained from each culture is presented.

Our aim was to find out what is the active component of the diffusate. From a number of different compounds tested we found that glycine appeared to be able to replace at least partially the diffusate. As can be seen in table 1, glycine significantly increased DNA synthesis in PHA stimulated lymphocytes, even in complete absence of serum components. In fact, glycine was able to replace almost half of the activity of diffusate. Table 1 also shows that triglycine was active to the same extent as glycine.

We were interested to know, what is the size of the glycine containing peptide which will be able to maintain the DNA synthesis at a similar level as in the presence of glycine (or triglycine). The experiment, described in fig.2, shows that there is a sharp decrease in DNA synthesis in cultures which contained tetraglycine. Thus, the borderline in the effective replacement of glycine by peptides, lies between triglycine and tetraglycine. Nevertheless some increase (as compared to controls) occurred in the presence of tetraglycine and at higher concentration, even with pentaglycine. The experiment described in fig.2 also shows, that triglycine could be replaced by L-leucyl-glycyl-L-leucine.

The results of these experiments suggested that nutritional factors, present in the diffusate, are responsible for the augmentation of the mitogenic effect of PHA. Since glycine was able to cover a considerable part of the diffusate, we speculated that perhaps other non-essential amino acids could, in addition to glycine, further increase DNA synthesis and even completely replace the diffusate. The experiment described in table 2 indicates that this was indeed the case. In this experiment Eagle's MEM was replaced by Dulbecco's medium (Flow Labs) which differs from the former by containing all non-essential amino acids, (e.g., glycine (30 μg/ml)). In this medium (the supplements being the same as described in Materials and methods) the same stimulation was obtained irrespective of further addition of glycine. Moreover, the diffusate could be here practically completely replaced by Dulbecco's medium. However, a further augmentation of the DNA synthesis could be obtained with the presence of the macromolecular components of the serum, i.e., the 40–65% ammonium sulfate fraction of the fetal calf serum.

Table 1
The ability of glycine or triglycine to partially replace the action of diffusate

Additions	– Diffusate (cpm × 10^{-3})	+ Diffusate
–	1.45 ± 0.2	18.85 ± 0.2
Glycine	8.30 ± 0.99	20.10 ± 0.14
Triglycine	9.60 ± 0.14	19.35 ± 2.90

Human lymphocytes were cultured in Eagle's MEM with supplements (see Materials and methods) in the absence of serum. The preparation of diffusate (5%, v/v) is described in fig.1. The concentration of glycine and triglycine was 85 μg/ml. In all cultures phytohemagglutinin (1 μl/culture) was present. The mean value of double cultures with standard deviation are presented.

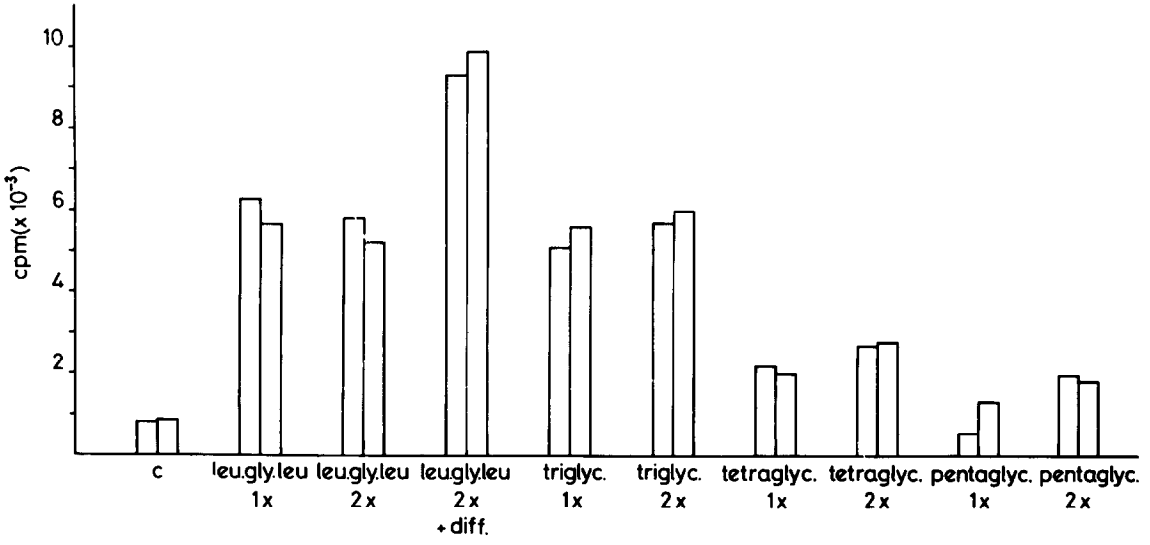

Fig.2. Effect of glycine peptides on DNA synthesis of human blood lymphocytes stimulated by phytohemagglutinin. Human lymphocytes were cultured in Eagle's MEM with supplement (see Materials and methods) in the absence of serum. The concentration of the peptides was 85 μg/ml (1×) and 170 μg/ml (2×). In the control cultures (c) no peptide was present, everything else being the same as in the experimental groups. In all cultures phytohemagglutinin was present. The value obtained from each culture is presented.

Table 2
The ability of Dulbecco's modification of Eagle's medium to replace the diffusate

Additions	– (cpm × 10^{-3})	Diffusate	Fraction 2
–	11.55 ± 2.47	14.75 ± 0.1	37.92 ± 1.92
Glycine	11.75 ± 1.62	16.0 ± 1.41	38.27 ± 1.41
Triglycine	12.65 ± 1.77	15.0 ± 2.83	38.12 ± 2.76
Diffusate	–	–	36.82 ± 3.61

Human lymphocytes were cultured in Dulbecco's modification of Eagle's medium with supplements (see Materials and methods) in the absence of serum. The concentration of diffusate and fraction 2 was 5% (v/v). (For preparation of fraction 2 see fig.1 'Fr.2'.) In all cultures phytohemagglutinin (1 μl/culture) was present. The concentration of glycine and triglycine was 85 μg/ml. The mean value of double cultures with standard deviation are presented.

4. Discussion

The mitogenic stimulation of lymphocytes is usually carried out in the presence of serum. The role which the serum plays in these cultures is not known. The experiments, described here, indicate that under certain experimental conditions the presence of glycine or its peptides and possibly other non-essential amino acids were of absolute necessity for the stimulation of human lymphocytes by phytohemagglutinin and that these could be supplied by fetal calf serum. Thus, we conclude that the supply of nutritional factors could be at least one mode of action by which serum could effect this process,

provided these factors are absent in the basal medium. If, however, these nutritional factors are present in the medium, the process of the stimulation of lymphocytes by the phytohemagglutinin will take place even in the complete absence of serum. These observations support the view that, for lymphocyte stimulation by phytohemagglutinin, the presence of serum in the medium is not absolutely necessary. Our results, however, also show that, under our experimental conditions, an optimal stimulation could be obtained only if, in addition to the nutritional factors, serum proteins were present in the system. These proteins could be separated from the serum by ammonium sulfate fractionation. Their role in the process of stimulation is unknown.

Kirchner and Oppenheim [3] found that chicken lymphocytes were stimulated by mitogens in the absence of serum. A similar result was obtained by Gazit and Harris [4] with leukocyte cultures of mouse spleen cells. Waithe et al. [5] have found that the stimulation of lymphocytes by PHA could proceed normally even in the absence of non-essential amino acids. However, these authors used protein synthesis for assessing lymphocyte stimulation and it is possible that this is the sole source for the discrepancies between their and our results. In such a case, the possibility is emerging that while the glycine demand for increased protein synthesis in the G1 phase could be covered by cellular synthesis, this is being not the case for purine synthesis in the S phase.

The fact that glycine could be replaced by its peptides suggests that an efficient peptidase is acting in this system. The activity of this enzyme system was not rate limiting even if leu–gly–leu was used. On the other hand, a sharp drop in the efficiency of the peptides was observed when higher peptides than tripeptides were used. We assume that this is due to lower permeability of the cells towards higher peptides than tripeptides.

Peters and Hausen [6] found that the membrane changes, known to occur after addition of PHA to cells, include increased 'faciliated diffusion' of sugars and uridine. Similarly an increase of amino acid uptake was observed in lymphocytes, stimulated by the mitogen [7]. The observation, that the borderline between utilisable and non-utilisable peptides lies between tri- and tetrapeptides, raises the question whether the pore size is affected during the permeability changes in stimulated lymphocytes. The system described here could provide a useful tool for elucidation of this problem.

Acknowledgement

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