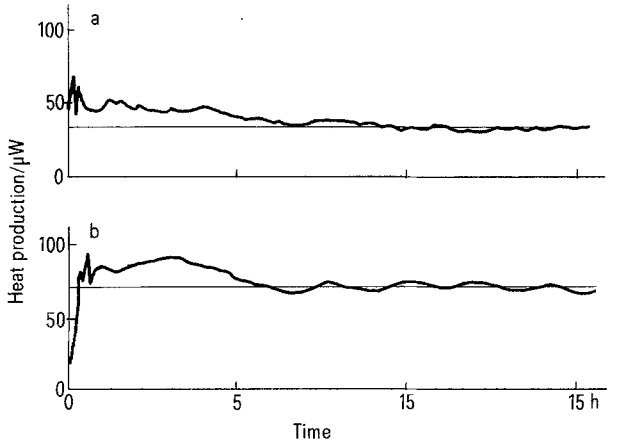

Power-time curve (thermogram) of human epidermal keratinocytes in a primary culture of normal cells with a final cell number of 4.2×10^5 (a) and in a culture of the transformed line SV-K14 with a final cell number of 5.5×10^5 (b).

phere) in a 1:1 mixture of DMEM and Ham's medium F12 containing 100,000 units penicillin, 100 mg streptomycin, and 250 µg amphotericin B per l of the medium which was supplemented with 5–10% fetal calf serum depending on the batch.

Preparation of the cultures for calorimetric experiments. After aspirating the culture medium the Petriperm™ dishes were placed upside-down under a laminar flow hood. The outer side of the bottom membrane was sterilized by the addition of absolute ethanol which within 1 min was completely removed by aspiration. Then the membrane was cut out with a sterile scalpel and placed aseptically with the cellular side inwards in a heat-sterilized calorimeter vessel made of stainless steel (17 mm diameter, 80 mm height). After slowly adding 10 ml DMEM (in which the sodium bicarbonate was replaced by 25 mM HEPES, pH 7.2), the calorimeter vessel was closed tightly, prethermostated at 35.5 °C for 15 min and introduced into a differential batch calorimeter (Bioflux from Thermanalyse, France). The reference vessel was treated in the same manner but charged with a blank Petriperm™ membrane. The calorimetric system was allowed to equilibrate for 20 min. Then the heat production rate was continuously recorded as a power-time curve (thermogram) at a sensitivity of 43.7 µV/mW. At the end of the experiments the Petriperm™ membranes were inspected microscopically.

Protein determination. The cells were extracted with 4 ml 5% (w/v) trichloroacetic acid and then 'solubilized' in 4 ml 0.5 N NaOH. To 100 µl of these solutions, 700 µl 0.071 N HCl and 200 µl Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, München, FRG) were added. The samples were processed for protein determination as described in the protocol supplied with the reagent. Bovine serum albumin, fraction V, was used as the standard.

Results and discussion. The keratinocyte is the principal cell type of the upper layer of the skin, the epidermis. Its replication takes place in the basal cell layer from which the cells move upwards, undergoing a distinct program of terminal differentiation leading to the dead horny layer. The time of transfer for one cell through the entire epidermis is about one month.

Normal keratinocytes in primary culture preserve most of their in vivo properties although their differentiation program is incomplete[4]. Confluent cultures consist of differentiating cells and those with basal cell character. Their proliferative activity, however, is very low as can be deduced from the few mitotic figures to be observed under the inverted phase-contrast microscope. The heat profile of such a culture is given in the figure (a). The fluctuations in the first hour are due to physical pertubations and thermal equalization. Within the next five hours, the power-time curve declines to a rather constant level representing an equilibrated energy metabolism. The increased heat production observed in the first phase of the experiment can be explained by enhanced metabolic activity due to the medium change and to the repair and replacement of cells which have been damaged by the transfer of the Petriperm™ membrane into the calorimeter vessel. From the plateau values of nine independent experiments a heat evolution (± SD) of (83 ± 12) pW/cell or (207 ± 30) mW/g protein has been calculated which is of the same order of magnitude as the values reported for other tissue cells[2,5,6].

Transformed cells are 'immortalized' and keep their proliferative capacity[7]. Even under confluent conditions a large number of mitotic figures can be seen. The surplus of cells detaches from the support and dies. The higher metabolic activity of the transformed cells is reflected in the higher heat output of (134 ± 35) pW/cell or (334 ± 87) mW/g protein (fig., b) which has been calculated from 11 independent experiments.

In conclusion, the described procedure using Petriperm™ tissue culture dishes provides a simple means for the calorimetric study of transformed and nontransformed anchorage dependent cells.

* To whom all correspondence and reprint requests should be addressed: Prof. Dr B. Schaarschmidt, Institut für Biophysik, Freie Universität Berlin, Thielallee 63, D-1000 Berlin West 33.

1 Kemp, R. B., Pestic Sci. *6* (1975) 311.
2 Schaarschmidt, B., and Reichert, U., Exp. Cell Res. *131* (1981) 480.
3 Liu, S.-C., and Karasek, M., J. Invest. Derm. *71* (1978) 157.
4 Holbrock, K. A., and Hennings, H., J. Invest. Derm. *81* (1983) 11s.
5 Ceretti, D. P., Dorsey, J. K., and Bolen, D. W., Biochim. biophys. Acta *462* (1977) 748.
6 Ljungholm, K., Wadsö, I., and Kjellén, L., Acta path. microbiol. scand. (B) *86* (1978) 121.
7 Taylor-Papadimitriou, J., Purkis, P., Lane, E. B., McKay, I. A., and Chang, S. E., Cell Diff. *11* (1982) 169.

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On the quaternary structure of *Kinixys erosa* hemoglobins

M. A. Ayeni and A. A. Aboderin

Department of Biochemistry, University of Ife, Ile-Ife (Nigeria), 25 January 1985

Summary. Kinixys erosa hemoglobins TH_I and TH_{II} comigrate on SDS-PAGE with mol. wt of 18 kD. Consistent with their amino acid compositions, trypsinolysis of the succinylated molecules and hydrolysis of cyanylated TH_{II} yielded five and two fragments respectively.

Key words. Chelonian hemoglobins; *Kinixys erosa;* quaternary structure; homomerism.

In earlier publications[1,2] the isolation and determination of some physicochemical properties of electrophoretically homogeneous hemoglobin fractions from red cell hemolysates of the testudinid *Kinixys erosa* were described.

On the basis of the data on amino acid composition, and the mol. wt values determined by the gel filtration method of Andrews[3], a proposal was made as to the possible homomeric nature of the quaternary structure of the two hemoglobin fractions TH_I and TH_{II}. In addition, the putative repeating unit or protomer, with a molecular weight of about 18 kD, is larger than the normal vertebrate α or β chain, and closer in size to the typical vertebrate myoglobin molecule, including that from the skeletal muscles of *K. erosa* itself[4].

Not a few of the major physiological characteristics of vertebrate hemoglobins e.g. the sigmoidal ligand binding curve, the Bohr effect and the mode of action of modulators such as 2,3-diphosphoglyceric acid have been attributed to the existence of a heteromeric molecule. Indeed the existence of a homomeric hemoglobin, at least in the case of humans, is associated with a pathological condition[5,6]. For this reason, and also because *K. erosa* is a representative of an order of reptiles *(Chelonia)* whose morphology appears to have remained unmodified since the initial divergence from the reptilian stock in the Permian some 250 My ago[6], it is of some interest to inquire further into the quarternary structure of these hemoglobin molecules. This will contribute to ideas about the evolution of respiratory globins[7].

Materials and methods. Diethylaminoethyl-(DEAE)-Sephacel, Sephadex G-50 and mol. wt standards (Bovine Serum Albumin 68 K, Ovalbumin 40 K, Chymotrypsinogen 25 K, Lysozyme 14.4 K and Horse heart Cytochrome C 11.5 K) were products of Pharmacia AB, Uppsala, Sweden. Bovine trypsin and Tris (trihydroxymethylamino methane) were products of Boehringer, Mannheim. Materials for gel electrophoresis and other routine chemicals of analytical grade were from British Drug House while paper for chromatography (Whatman 1) was a product of W&R Balston Ltd, Poole, England.

Kinixys hemoglobin fractions TH_I and TH_{II} and their corresponding globins were isolated as described in the earlier publication except that the initial resolution of the hemolysate was carried out using DEAE-Sephacel instead of DEAE-Cellulose.

Succinylation of TH_I and TH_{II} globins. The preparation of succinylated derivates of globins from TH_I and TH_{II} was achieved by an adaptation of the method of Klotz[8] as follows: To 30 mg of protein dissolved in 0.01 M pyrophosphate buffer, pH 8.5, containing 6 M guanidine hydrochloride was added 300 mg of solid succinic anhydride during a 50-min period. The pH of the reaction mixture was maintained at 8.5 by adding aliquots of 2 M NaOH as necessary. Total reaction time was 90 min, after which the reaction mixture was dialyzed exhaustively against the pyrophosphate buffer. The succinylated protein solution was then stored at 4 °C.

Enzymatic hydrolysis of succinylated globins. Succinylated globins prepared as described above were subjected to hydrolysis by bovine trypsin in 0.01 M pyrophosphate buffer pH 8.5 for 2 h at room temperature (25 °C) using a protein to enzyme ratio of 70:1. Hydrolysis was terminated by lowering the pH of the digest to 2 using concentrated hydrochloric acid.

Cyanylation of TH_{II} globin[9,10]. TH_{II} globin (400 mg, 2.2 µmoles) was dissolved in 3 ml of 0.2 M Tris acetate buffer containing 6 M guanidine hydrochloride. To the resulting solution was added 21 mg (0.14 mmoles) of dithiothreitol.

After 30 min at room temperature 280 mg (1.2 mmoles) of 2-nitro-5-thiocyanobenzoic acid, which had been prepared following the procedure of Degani and Patchornik[11], were added to the protein solution. The cyanylation reaction was for a further 15 min. The product was dialyzed exhaustively against 50% (v/v) acetic acid and then lyophilized.

Cleavage of cyanylated TH_{II} globin. Lyophilized cyanylated TH_{II} globin was dissolved in 4 ml of a solution containing 6 M guanidine hydrochloride and 0.1 M sodium borate. After adjusting the pH of the protein solution to 9.1, the mixture was allowed to stand at room temperature for 24 h. The resolution of the reaction products was carried out as described in the results section below.

Amino acid analysis. Amino acid analysis of peptide fragments were carried out on a Durrum D-500 Amino Acid Analyzer at the Institute of Marine Resources of the University of California, Davis, Cal., USA.

Results and discussion. 1) Molecular size of subunit/protomer. Figure 1 represents schematically the results of electrophoretic analyses of *Kinixys* hemoglobins in polyacrylamide in the absence (A) and presence (B) of sodium dodecyl sulfate.

The mobilities recorded in the absence of detergent are in agreement with the earlier observations on the charge characteristics of the two hemoglobin molecules while the pattern observed at the same time affirms the homogeneity and individuality of each of the two molecules in the organism.

The results obtained in the presence of detergent show that the subunits of TH_I and TH_{II} which co-migrate (fig. 1B) have the mobility to be expected of a molecule with a mol. wt of 18 kD (fig. 1C). This result is consistent with the previous inference from the amino acid compositional data[2] and with a quaternary structural stoichiometry of four identical subunits in the native ensemble when taken together with the previously determined molecular size of the un-denatured molecules.

2) Chemical evidence for the homomeric quaternary structure. Proteolytic fragmentation of polymeric proteins by agents of narrow and predictable cleavage specificities can provide information not only on the stoichiometry of the polymeric ensembles but also on the extent of the identity of the constituent subunits[13]. Advantage has been taken in this investigation of the existence of only four residues of arginine in the putative repeating units of TH_I and TH_{II} respectively[2], and of only one cysteine

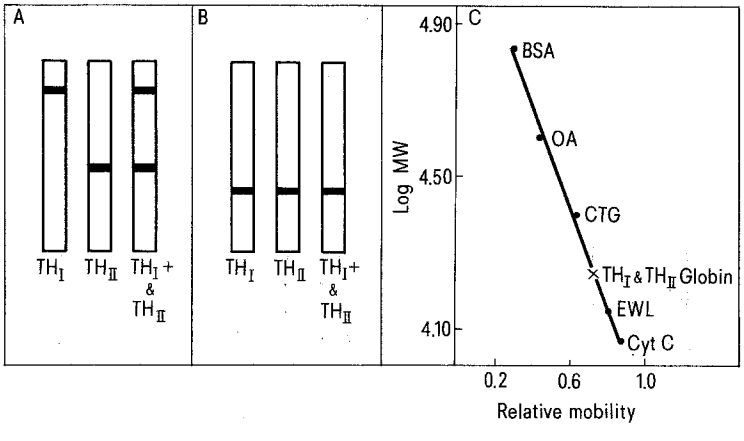

Figure 1. Polyacrylamide gel electrophoresis of TH_I and TH_{II} in the absence (A) and presence (B) of sodium dodecylsulfate. The mobilities of mol. wt standards (1: bovine serum albumin (BSA), 2: ovalbumin (OA), 3: chymotrypsinogen (CTG), 4: hen egg white lysozyme (EWL), and 5: horse heart cytochrome C (CytC)) and of TH_I and TH_{II} determined in 7.5% gels in the presence of detergent according to the method of Weber and Osborne[12] are summarized in (C).

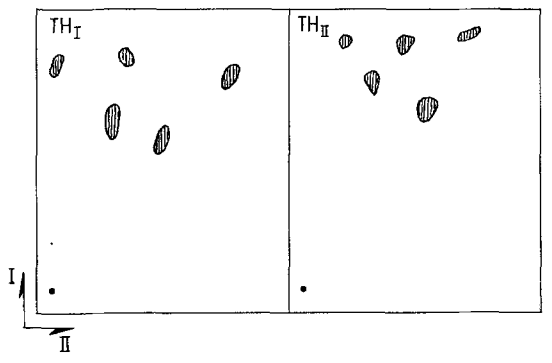

Figure 2. Schematic representation of two-dimensional paper chromatograms of the products of tryptic hydrolysis of succinylated TH_I snf and TH_{II} globins, Solvent I was phenol/butanol/acetic acid/water (3:3:2:4 (v/v)); solvent II was n-butanol/acetic acid/water (4:1:5 (v/v)); chromatograms were stained with a 0.1% ninhydrin solution in ethanol/acetic acid/collidine (60:20:8 (v/v).

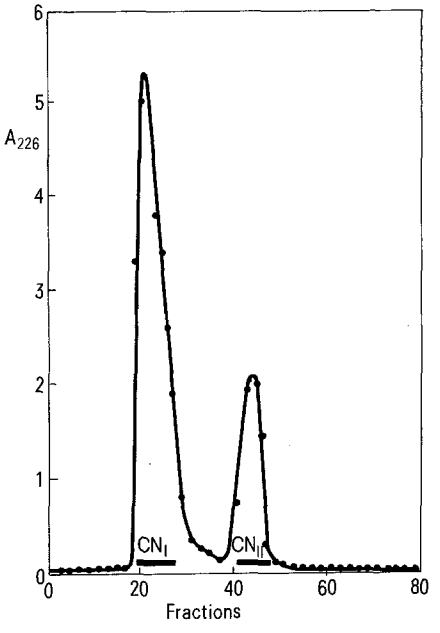

Figure 3. Gel filtration in Sephadex G-50 (90 × 1.5 cm) of the products from the hydroxide-promoted cleavage of cyanylated TH_{II} globin. Column was eluted with 5 mM HCl. Fraction size was 3 ml. Flow rate was 30 ml/h. CN_I and CN_{II} were pooled as indicated.

residue in the repeating unit of TH_{II} for the choice of cleavage conditions: trypsinolysis of succinylated TH_I and TH_{II} globins and mild alkaline hydrolysis of cyanylated TH_{II} globin.

The hydrolysis by trypsin of succinylated globins should lead to the production of five peptides (or four, in the event that one of the arginyl residues is to be found at either the N or the C terminal position of the putative repeating unit) whilst in the case of the mild alkaline hydrolysis of the cyanylated TH_{II} globin which will occur only at the amino end of the modified cysteinyl residue[9,10] two peptide fragments should be the result if the hypothesis of a homomeric ensemble for both hemoglobin molecules is correct.

Figure 2 is a schematic representation of two dimensional paper chromatographic separation experiments on the tryptic peptides produced from succinylated globins from TH_I and TH_{II}. The chromatograms bear a remarkable similarity to each other – a possible expression of extensive primary structural homology. In both cases only the theoretically expected five ninhydrin-positive fragments have been obtained.

Figure 3, on the other hand, is the resolution on Sephadex G-50 of the reaction mixture following mild alkaline hydrolysis of cyanylated TH_{II} globin[9]. Aliquots of the two fragments CN_1, and CN_2, which were further purified by a second chromatographic run using the same conditions as were utilized for the initial separation, were subjected to acid hydrolysis and the amino acid composition of each was determined.

The amino acid compositions of the two fragments are summarized in the table, which also contains the data on TH_{II}[2]. Although there are some differences in the molar ratios of some amino acids (notably Val, Ile and Leu) when compared with the data on TH_{II}, the overall picture – and the fact that over 96% of the expected amino acid residues in TH_{II} are accounted for in the cumulative results from CN_1 and CN_2 – is consistent with the position that TH_{II} is a homomeric ensemble in the native state. From the data presented in this and in previous work[1,2], it is to be concluded that *Kinixys erosa* possesses two hemoglobin molecules, both of which are homotetramers. The occurrence of such molecules in what is to be considered as a normal physiologically functioning organism can only mean that a heterotetramer may not always be a structural prerequisite for vertebrate hemoglobin function[6]. Further work will be necessary to delineate clearly the nature of the ligand-binding characteristics of these hemoglobins as well as the identity and mode of action of the modulators of the binding of ligand that are native to *Kinixys*.

Amino acid composition of TH_{II} and its cyanylation fragments

Amino acid	CN_I molar ratio[a]	Nearest integer	CN_{II} molar ratio[b]	Nearest integer	$CN_I + CN_{II}$	TH_{II}[c]
ASP	11.02	11	2.63	3.0	14	13
THR	4.70	5	1.83	2	7	7
SER	5.64	6	2.56	3	9	9
GLU	11.50	12	3.39	4	16	16
PRO	3.22	3	1.18	1	4	5
GLY	5.19	5	2.04	2	7	7
ALA	11.05	11	4.29	4	15	16
VAL	7.58	8	3.78	4	12	14
MET	0.91	1	–	0	1	1
ILE	3.05	3	0.85	1	4	5
LEU	17.00	17	4.18	4	21	19
TYR	2.96	5	1.09	1	4	4
PHE	5.22	5	1.71	2	7	8
HIS	9.12	9	2.13	2	11	11
LYS	6.72	7	2.75	3	10	11
ARG	2.98	3	0.92	1	4	4
TRP	ND	ND	ND	ND	ND	

[a]To determine the molar ratios of the amino acid residues, 8.50 nmoles of peptide were hydrolyzed for 24 h in 1.0 ml of constant boiling HCl. [b]18.64 nmoles of CN_{II} were hydrolized as described above. [c]From Aboderin and Obidairo[1]. ND, not determined.

In addition, the elucidation of the possible evolutionary mechanisms which will account for the existence of what is an abnormal homomeric condition in a vertebrate hemoglobin will depend on the availability of the full primary structural information on both TH_I and TH_{II}, both of which are most likely to have extensive structural similarities (fig. 2), and on similar studies on other members of the Testudinidae namely *Gopherus, Homopus, Malacocherus,* and *Testudo* spp.

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1 Aboderin, A. A., and Obidairo, T. K., Comp. Biochem. Physiol. (BV) *54* (1976) 417.
2 Obidairo, T. K., and Aboderin, A. A., Experientia *35* (1979) 22.
3 Andrews, P. E., Biochem. J. *96* (1965) 595.

4 Okotore R. O., and Aboderin, A. A., Comp. Biochem. Physiol. (B) *62* (1979) 567.
5 Antonini, E., and Brunori, M., Hemoglobins and Myoglobins in their Interaction with Ligands. Elsevier, New York 1971.
6 Benesch, R., and Benesch, R. E., Science N.Y. *185* (1974) 405.
7 Young, J. Z., The Life of Vertebrates, chap. XIV. Oxford University Press, Oxford 1962.
8 Klotz, I. M., in: Methods in Enzymology, vol. 2, p. 576. Ed. C. H. W. Hirs. Academic Press, New York 1967.
9 Jacobson, G. R., Schaffer, M. H., Stark, G. R., and Vanaman, I. C., J. biol. Chem. *248* (1973) 6583.
10 Degani, Y., and Patchornik, A., Biochemistry *13* (1974) 1.
11 Degani, Y., and Patchornik, A., J. org. Chem. *36* (1971) 2727.
12 Weber, K., and Osborne, M., in: The Proteins, vol. 1, 3rd edn, p. 179. Eds Neurath and R. L. Hill. Academic Press, New York 1975.
13 Klotz, I. M., Darnall, D. W., and Langerman, N. R., in: The Proteins, vol. 1, 3rd edn, chap. 5. Eds H. Neurath and R. L. Hill. Academic Press, New York 1975.

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Cortisol and immune interferon can interact in the modulation of human natural killer cell activity[1]

R. Cavallo, M.L. Sartori, G. Gatti and A. Angeli[2]

Istituto di Medicina Interna, Cattedra di Patologia Medica D, Università di Torino, Strada S. Vito, 34, I-10133 Torino (Italy),
28 January 1985

Summary. This paper reports that cortisol at physiological concentrations minimizes the enhancement of human natural killer (NK) cell activity in vitro by immune interferon (IFN-γ). This effect may be important for the regulation of NK cytotoxicity in vivo.
Key words. Natural cytotoxicity; cortisol; immune interferon.

Natural killer (NK) cells are a heterogeneous subpopulation of lymphocytes capable of lysing target cells without prior sensitization. Since NK cell activity is most evident against neoplastic and virus-infected targets, it is thought that NK cells in vivo play an important role in immunosurveillance[3,4]. Recently, a number of studies have been carried out in order to understand the physiological mechanisms by which NK cell activity is regulated. Evidence has been provided that interferon (IFN) preparations and IFN inducers enhance basal NK cell activity and counteract the effects of pharmacological agents which have been shown depress NK cell cytotoxicity[5,6]. Synthetic glucocorticoids, on the other hand, have been found to be inhibitors of rodent and human NK cell activity[7,8] and to suppress at high concentrations the enhancement induced in vitro by leukocyte type I interferon (IFN-α)[9].

On the basis of these reports, we thought it pertinent to investigate whether cortisol, the most representative endogenous glucocorticoid in man, and the immune interferon (IFN-γ), a glycoprotein produced by T-lymphocytes and more closely associated with immunosurveillance than other IFN preparations, can interact in the modulation of human NK cell activity.

Materials and methods. PBM cells were derived from heparinized venous blood samples obtained from healthy adult donors (males and females, aged 20–35 years), taking no medication, and fasting for 8 h before venipuncture. PBM cells were immediately separated by Ficoll-Hypaque density centrifugation[10]. The resultant preparations contained more than 98% mononuclear cells. PBM cells were washed and resuspended to the desired density in complete medium, which is the medium RPMI 1640 supplemented with 10% foetal calf serum (Eurobio, Paris, France), 1% L-glutamine and gentamicin (50 μg/ml).

Cortisol (Sigma Chemical Co., St. Louis, Missouri, USA) was initially dissolved in a little 95% ethanol and then diluted in distilled water to a 1×10^{-3} M stock solution. For use in experiments, this preparation was promptly diluted in distilled water to a final concentration ranging from 1×10^{-5} to 1×10^{-8} M. We have documented that NK cytotoxicity of PBM cells against K 562 targets was significantly reduced by cortisol at the above-reported range[11]. Highly purified human IFN-γ (generously provided by F. Dianzani, Institute of Virology, University of Rome, Italy), diluted in RPMI 1640 was used in the range from 5 to 30 IU/ml. Preliminary experiments on the dose-response curve within this range showed the 15 IU/ml concentration to give an optimal enhancement of NK cell activity. The effects on NK cell activity of treatment with either cortisol or IFN-γ require a relatively long period of time to become manifest. In the present series of experiments, PBM cells, resuspended in complete medium to a density of 3×10^6 cells/ml, were incubated for 20 h in the presence of cortisol and/or IFN-γ at 37°C in a humidified atmosphere of 95% air and 5% CO_2. We carried out the majority of experiments using cortisol at the concentration of 1×10^{-6} M and IFN-γ at the concentration of 15 IU/ml. No significant differences in viability were observed after in vitro incubation of PBM cells with both agents, in comparison with untreated PBM cell preparations.

NK cell activity was determined in a 4-h direct ^{51}Cr-release assay as previously described[12]. The human cell line K 562 was used as the source of target cells. Percentages of NK cell-mediated cytotoxicity were computed from the following formula:

$$\%\text{cytotoxicity} = \frac{\text{cpm (test)} - \text{cpm (spontaneous)}}{\text{cpm (total)} - \text{cpm (spontaneous)}} \times 100$$

Test cpm, total cpm and spontaneous cpm represent the radioactivity released in the supernatants from target cells incubated with effectors, in an equal aliquot of target cells in suspension and in supernatants from target cells incubated without effector cells, respectively. Spontaneous release never exceeded 10% of the maximum release. The variations among triplicates were less than 5%.

Except where indicated, data were expressed as lytic units (LU)/10^7 effector cells. The LU values were derived from equation $y = A(1-e^{-kx})$ where y = fractional chromium release, A = a constant equal to the asymptote of the sigmoid curve, k = a constant proportional to effector cell activity for a given A and x = the E:T ratio. In these experiments 1 LU represents the number of cells required to give a 30% cytotoxicity.