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Iron Compounds after Erythrophagocytosis: Chemical Characterization and Immunomodulatory Effects

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In humans, the lymphomyeloid system has a fundamental role on iron metabolism promoting its recycling due to a continuous removal of effete red blood cells. Additionally, one of the most intriguing aspects of metalloporphyrins in biology is their effect on the immune system. However, the process of erythrocyte catabolism is still poorly understood and needs further research. In the present study, we attempt to investigate the nature and the possible physiologic role of Fe compounds released after erythrophagocytosis during the removal of red blood cells. Monocyte erythrophagocytosis *in vitro* experiments were done to characterize chemically the Fe compounds present inside the cells and in the culture supernatants. We tested the probable immunomodulatory functions of erythrophagocytosis products over lymphocyte cultures activated *in vitro* with T mitogens (α-CD3). Data obtained from atomic absorption spectroscopy confirmed the presence of Fe in the culture supernatants of monocyte cultures after erythrophagocytosis. Also, high-spin haem complexes derived from erythrocyte catabolism were detected by electron paramagnetic electronic resonance. Finally, *in vitro* activated lymphocyte proliferation experiments indicate the co-mitogenic properties of monocyte culture supernatants after red blood cells phagocytosis. Thus, the results of the present work provide evidence that culture monocyte supernatants after *in vitro* erythrophagocytosis contain Fe (III) high-spin haem complexes and show lymphocyte proliferation co-stimulatory properties. © 1998 Academic Press

Key Words: **iron; haem; erythrophagocytosis; ferric mitogens.**

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The cells of the mononuclear-phagocytic system (MPS) play a central role in Fe metabolism specially due to their involvement in erythrocyte catabolism [1]. In normal conditions, the MPS provides most of the Fe required for erythropoiesis, Fe storage and release by the MPS are in equilibrium. In this way, haemoglobin synthesis appears to be exclusively dependent on MPS regulation and autonomous from the external Fe supply [1,2]. Iron has been suggested to contribute to the modulation of the immune response [3-7]. In fact, it was documented that iron (as a product of red cell breakdown and recycling) can be regarded as a target of immune surveillance and may also be considered as having a direct modelling effect on the immune system [8].

Since cells of MPS are central to the removal of senescent red blood cells this continuous process of recycling is one of the most significant illustrations of the interaction between iron and the immune system [9]. Moreover, the influence shared by these two systems can be further extended by the possibility of immunomodulatory functions by released erythrophagocytosis products.

Previous studies have documented the immune stimulatory properties of metalloporphyrins [10] including iron-containing compounds [5,6,11] and haem complexes [10,12,13]. For example, haemin is recognized as a mitogenic and co-mitogenic agent for human peripheral blood mononuclear cells (PBMC) [10,12-14] acting sinergestically with IL-2 on the induction of mitogenicity, citotoxicity and cytokines release by these cells [10,12]. Furthermore, it is well known the lymphocyte-stimulatory properties of different "Ferro-mitogens" including horseradish peroxidase, cytochrome c, myoglobin and transferrin [11,12,15].

In this paper we report the chemical nature of iron compounds present in monocyte culture supernatants (SN) after erythrophagocytosis *in vitro* and show their co-mitogenic properties.

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TABLE I

EPR Results from Red Blood Cell Samples

Sample[1]	[RBC/ml][2]	EPR g = 6 signal[3]
R1	1×10^6	−
R2	1×10^7	−
R3	1×10^8	++
R4	1×10^9	+++

[1] R1/R2/R3/R4- Red blood cell control samples.
[2] Erythrocyte concentration (number of RBC/ml).
[3] − not detected, ++ medium, +++ strong, g = 6 type EPR signal.

MATERIALS AND METHODS

Isolation of cells and culture conditions. Fresh peripheral blood mononuclear cells (PBMC) were obtained from buffy coats from healthy, normal subjects from the Blood Bank of Hospital Geral de Santo António (Porto) after centrifugation over lymphoprep (Nycomed, Oslo, Norway). PBMC were then washed twice in Hanks' Balanced Salt Solution (HBSS, Gibco Paisley, U.K.) and resuspended in RPMI 1640 complete medium (Sigma Chemical Co, St. Louis, U.S.A.) supplemented with penicillin (500 U.I./ml, Astral, Portugal), streptomycin (0.5mg/ml, Sigma), Hepes buffer solution (24 μM, Sigma) and heat-inactivated fetal calf serum (10% FCS, Sigma Co, St.Louis, USA). Partially purified (usually >80%) peripheral blood monocytes and lymphocytes were separated by conventional adherence techniques [26]. Human red blood cells (huRBC) were isolated by lymphoprep density gradient centrifugation and washed three times (635g, 5 minutes, 4 °C) with sodium citrate (13mM-PBS, pH 7.2) and one time with RPMI 1640 complete medium. HuRBC were opsonised after 30 minutes incubation (37°C water bath) with 1:2000 dilution of anti-huRBC rabbit (a kind gift from Prof. H.Dÿk, Eijkman Institute for Microbiology, Infectious Diseases and Inflamation, University Hospital Utrecht, Utrecht, The Netherlands).

Erythrophagocytosis. Monocytes previously isolated were incubated with opsonized huRBC (dilution 1:200) for 90 minutes at 37°C in a 5%CO_2 atmosphere. Not phagocytosed RBC were removed by hipotonic lysis with 0.2% NaCl solution during 16 seconds. The reaction was stoped by addition of 3% NaCl solution. Cells were washed twice with RPMI 1640 complete medium. Cell viability, as determined by Trypan blue exclusion, was always >85%. Phagocytosis was evaluated by optic microscopy of citospin stained with May-Grünwald-Giemsa (Dade diff-Quick solutions, Baxter, Düdingen, Switzerland).

Release of iron. After erythrophagositosis, monocytes (1×10^6 cells/ml) were plated in 12 well flat-bottomed culture plates (Nunclon, Denmark) and incubated for 24h at 37°C in a 5%CO_2 atmosphere and 95% humidity. Monocytes that had not been previously incubated with erythrocytes, but that for the rest were submitted to the same experimental conditions were used as controls. Supernatants from control cultures (SN_{24h}-C) and from cultures of monocytes after erythro-phagocytosis (SN_{24h}-R) were obtained after centrifugation at 660g, 4°C for 7 minutes. Cells still adherent to the plates were removed with distilled water and added to their respective cell fractions (pellet of the 660g centrifugation step, P_{24h}-C, P_{24h}-R). Fractions SN_{0h} and P_{0h} for control and monocyte cultures after erythrophagocytosis were obtained just before the incubation period (t=0h) following the same protocol. All samples were frozen at −70°C for further studies.

Iron compounds after erythrophagocytosis-quantitative and qualitative analysis. Iron content of supernatants was measured by atomic absorption spectroscopy (4100 ZL Perkin-Elmer). Redox state and molecular forms of iron present on SN and P fractions of cultures after erythrophagocytosis were evaluated by electronic paramagnetic resonance (EPR). Different erythrocyte dilutions, SN and P fractions from monocyte cultures that were not incubated with RBC were used as controls. All the spectra (X-band EPR) were obtained in a Bruker Instrument, at 8K, 3450 G (central field), 4000 G (observation window), 10 Gpp (amplitude modulation) after 3 scans.The g=6 signal (indication of haem high-spin species) was evaluated in a qualitative way denoted as strong (+++), medium (++), weak (+) or (−) not detected.

Proliferation assays. PBMC (1×10^6 cells /ml) were plated in 96 wells (50μl/well) flat-bottomed microtitre plates (Nunclon Delta, Denmark). Cultures were activated with monoclonal antibody anti-CD3 (Dakopatts M756, Copenhagen, Denmark) at a final dilution of 1:500 in RPMI. Substitution of anti-CD3 by RPMI 1640 (Sigma Chemical Co, St. Louis) was done in order to obtain activation control cultures. Increasing volumes (25μl, 50μl and 100 μl) of supernatant monocyte cultures after erythrophagocytosis were added to activated and not activated PBMC cultures. Supernatants from monocyte cultures not previously incubated with RBC were used to obtain super-

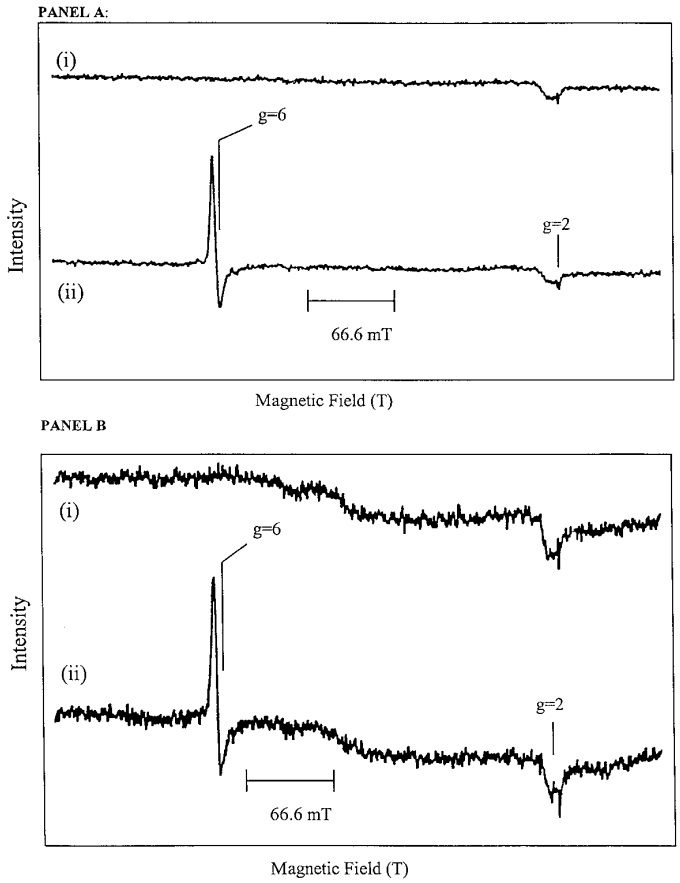

FIG. 1. EPR spectra from monocyte cultures after erythrophagocytosis and respective controls. **Panel A**- (i) Erythrocyte samples (concentration less than 1×10^7cells/ml). (ii) Erythrocyte samples (concentration greater than 1×10^7cells/ml (see Table I). **Panel B**- (i) Monocyte control cultures samples (isolated at t=0h and after 24h), and monocyte pellet isolated 24h after erythrophagocytosis (ii) Cell suspensions (T), supernatants (SN) and pellets (P) from monocyte cultures after erythrophagocytosis (t=0h), and samples T and SN from the same cultures after 24h incubation (see Table II). Experimental conditions: Temperature: 8 K; Central field: 3450 G; Observation window: 4000 G; Amplitude modulation: 10 Gpp; scans: 3.

TABLE II

EPR Spectra from High-Spin Haem Fe(III) Compounds on Monocyte Cultures after Erythrophagocytosis and Monocyte Control Cultures

	Time after erythrophagocytosis			
	0 h		24 h	
	Erythrophagocytosis[1]	Control[2]	Erythrophagocytosis[1]	Control[2]
Cell suspension	++++	−	++++	−
Pellet	++	−	−	−
Supernatant	+	−	++	−

Note. +++ Strong, ++ Medium, + Weak, − Not detected, g = 6 EPR type signal.
[1] Monocyte cultures after erythrophagocytosis.
[2] Monocyte control cultures (MN cultured in the same conditions except for the addition of RBC).

natant control cultures (activated and not). The final volume obtained in all cultures was 200μl/well. Cultures were incubated for 24h, 48h and 72h at 37°C in a 5%CO_2 atmosphere with 95% humidity. [^{3}H] Thymidine (Amersham, Buckinghamshire, U.K.) (0.2 μCi/well) was added 4h prior to the end of the incubation. [^{3}H]-TdR incorporation into DNA was measured using a scintilation counter. Cultures were performed in triplicate and the means determined.

Supernatant volume titration. A supernatant volume titration was done in order to determine the less volume of monocyte culture supernatant after erytrophagocytosis still able to show lymphocyte proliferation stimulatory effect. Different volumes of supernatants from monocyte cultures after erythrophagocytosis (0 μl, 1μl, 5μl, 10μl, 25μl, 50μl e 100μl /well) were added to PBMC cultures (not activated and activated with 1:500 anti-CD3 monoclonal antibody dilution). Equal volumes of supernatants from monocyte control cultures were used in order to obtain activated and not activated control cultures. Proliferation was accessed using the same protocol described before.

Co-stimulatory effects of supernatants from monocyte cultures after erythrophagocytosis. In order to make a better evaluation of the comitogenic properties of supernatants from monocyte cultures after erythrophagocytosis lymphocyte proliferation assays were performed in the presence of decreasing concentrations of anti-CD3 monoclonal antibody (dilutions 1:500, 1:1000, 1:5000 and 1:10000). PBMC (1×10^6 cells /ml; 50μl/well) were plated with MN supernatants (25 μl/well) in 96 wells flat-bottomed microtitre plates.

Cultures not activated with anti-CD3 and supernatants from monocyte cultures not incubated with RBC were used as controls. Proliferation was accessed using the same protocol described before.

IL-1β and TNF-α quantification. IL-1β and TNF-α were measured on supernatants from MN control cultures and MN cultures after erythrophagocytosis using ELISA. assays (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).All measurements were performed in duplicate.

RESULTS

Iron Compounds after Erythrophagocytosis

Quantitative analysis. Purified monocytes were cultured in the presence or absence of opsonized huRBC for 90 min. After lysis of nonphagocytosed RBC, MN were further incubated in RPMI for 0h and 24h and supernatants and pellets were collected separately. Iron in the supernatants was measured by atomic absorption spectroscopy. Iron concentration (μg/ml) is increased in supernatants from monocyte cultures after erythrophagocytosis comparatively to supernatantes from monocyte control cultures (144.3 (53.4) ν 7.0 (1.0), average (SD) of n=3 experiments).

Qualitative analysis. EPR spectra from hemolysed erythrocyte dilutions (red blood cells control) showed a typical signal of haemic systems in which iron is in a ferric high-spin form (g=6 and g=2) (Table I; Fig.1A). This signal was only detected on samples with concentrations greater than 1×10^7 RBC/ml. Table II shows EPR results from monocyte cultures after erythrophagocytosis and MN control cultures. Signal g=6 and g=2 was detected on MN cultures after erythrophagocytosis (Fig.1B, (ii) spectra) but not on MN cultures that had

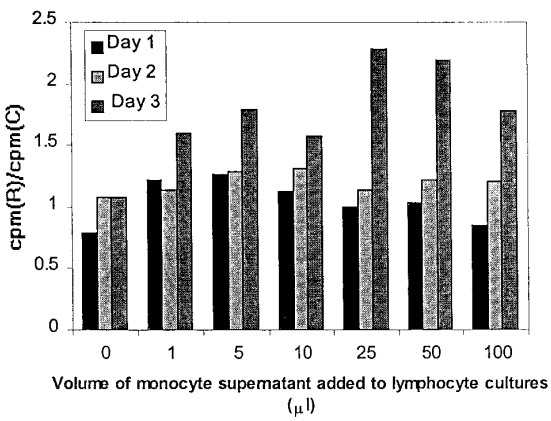

FIG. 2. Volume titration of supernatants from monocyte cultures used on T cell proliferation assays. cpm (R)- Average T cell proliferation obtained in the presence of supernatants from monocyte cultures after erythrophagocytosis; cpm (C)- Average T cell proliferation obtained in the presence of supernatants from monocyte control cultures; cpm(R)/cpm(C)- Ratio between average T cell proliferation obtained in the presence of supernatants from monocyte cultures after erythrophagocytosis and lymphocyte proliferation obtained in the presence of control supernatants.

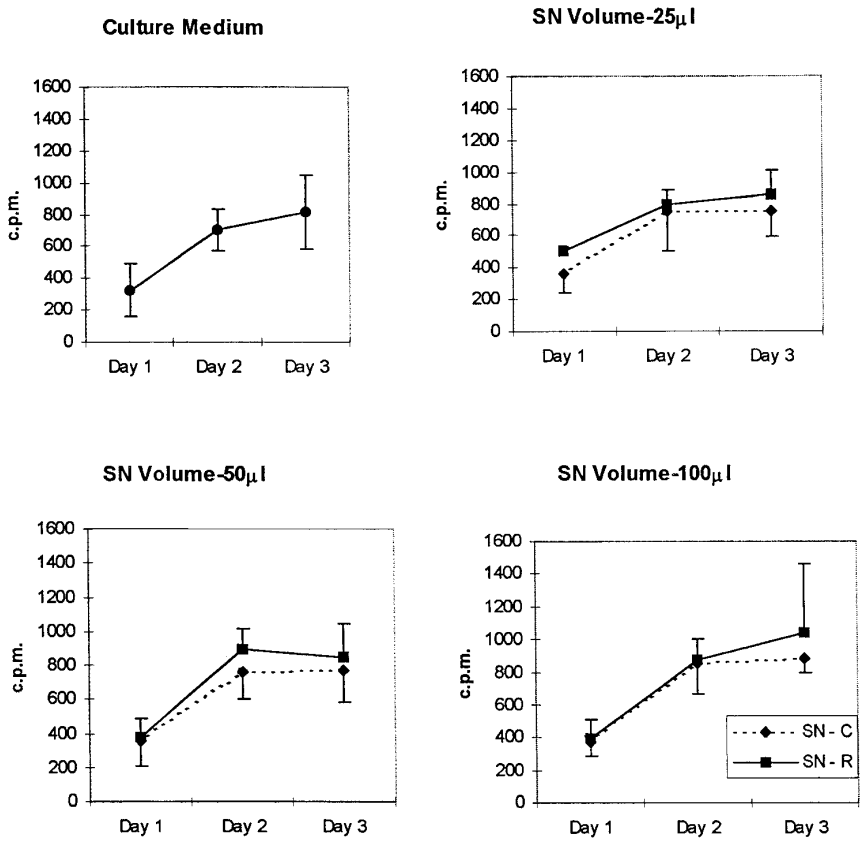

FIG. 3. Non activated T cell proliferation obtained in the presence of culture media, supernatants from monocyte cultures after erythrophagocytosis (SN-R) and supernatants from monocyte control cultures (SN-C). Results are expressed as average T cell proliferation (cpm)± SD, n=3.

not been incubated with RBC (Fig.1B, (i) spectra). The relative intensity of g=6 and g=2 signal was greater in total cell suspension than in their respective supernatants. Immediately after erythrophagocytosis (0h) monocyte pellets showed the typical EPR signal (Table II, Fig.1B, (ii) spectra). However, 24h after incubation the haemic high-spin Fe(III), g=6 and g=2 signal is not detected in pellets but only in the respective supernatants.

Immunomodulatory Effects of Monocyte Culture Supernatants after Erythrophagocytosis

Effect of monocyte culture supernatants on lymphocyte proliferation. In order to study the effect of monocyte culture supernatants on lymphocyte proliferation, supernatants from monocyte cultures previously incubated (cpm(R)) or not (cpm(C)) with eritrocytes were added to lymphocyte cultures at different concentrations (Fig.2). The greater immunologic T response was detected when 25 μl/well monocyte culture supernatant after erythrophagocytosis was used.

Co-stimulatory effects of supernatants from monocyte cultures after erythrophagocytosis. In order to test whether proliferative effect of erythrophagocytosing monocyte supernatants was able to potentiate the anti-CD3 mitogenic effect of T lymphocytes, in another set of experiments T lymphocytes were cultured in the presence of of anti-CD3 monoclonal antibody and addition of different concentrations of monocyte cultures supernatants.

Monocyte culture supernatants after erythrophagocytosis did not show T cell proliferation stimulatory properties when added to not activated T cell cultures (Fig.3). However, proliferation of anti-CD3 activated T cells in the presence of monocyte culture supernatants after erythrophagocytosis was increased compared to proliferation of the same cells in the presence of supernatants from monocyte cultures that did not phagocytose red blood cells (Fig.4).

In order to study whether the co-stimulatory effect of monocyte supernatants was also observed with high dilutions of anti-CD3 antibody, T cells were cultured in the presence of different dilutions of anti-CD3 antibody and in the presence of monocyte supernatants. The results depicted in Fig. 5 show that the co-stimulatory effects of monocyte culture supernatants after erythrophagocytosis were still present even when very high dilutions of anti-CD3 monoclonal antibody (1:1000 and 1:5000) were used.

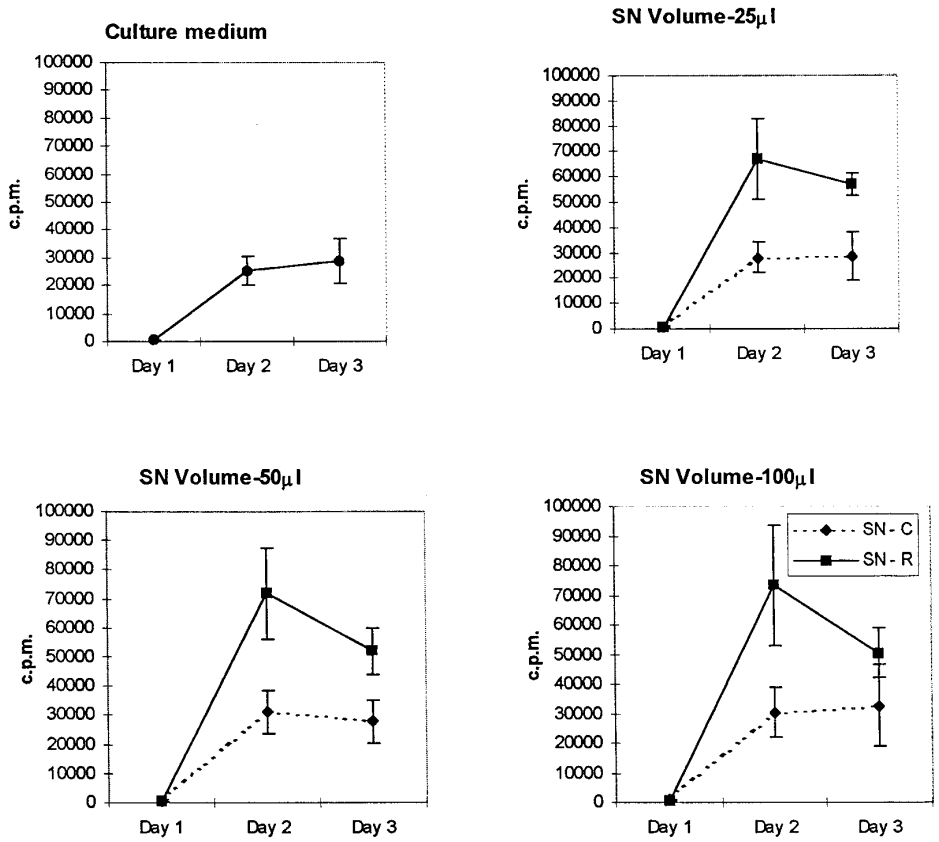

FIG. 4. Anti-CD3 (1:500 dilution) activated T cell proliferation obtained in the presence of culture media, supernatants from monocyte cultures after erythrophagocytosis (SN-R) and supernatants from monocyte control cultures (SN-C). Results are expressed as average T cell proliferation (cpm)± SD, n=3.

IL-1β and TNF-α Quantification

In order to investigate a possible role of cytokines on the immunomodulatory properties of supernatants from monocyte cultures after erythrophagocytosis IL-1β and TNF-α concentrations were determined in supernatants of monocyte cultures after erythrophagocytosis and control supernatantes. The results showed that TNF-α concentration (pg/ml) was increased in supernatants of monocyte cultures after erythrophagocytosis compared to controls (222.5 (111.0) ν 38.5 (20.3), average (SD) of n=4 experiments). However, IL-1β concentration was similar in both types of samples (760.0 (103.2) ν 778.0 (88.6), average (SD) of n=4 experiments).

DISCUSSION

In the present study monocyte erythrophagocytosis *in vitro* experiments were done in order to characterize chemically the iron compounds present inside the cells and on the culture supernatants. Furthermore, we tested the probable immunomodulatory properties of erythrophagocytosis products over lymphocyte cultures activated *in vitro* with T mitogens (α-CD3).

Results from atomic absorption spectroscopy and EPR spectroscopy revealed the presence of high-spin haem [Fe(III)] in monocyte culture supernatants 24h after erythrophagocytosis. Identical EPR spectra from red blood cell controls and supernatants from monocyte cultures after erythrophagocytosis suggest the presence of the same iron molecular form. The relative intensity of g=6 and g=2 signal was greater in total cell suspension at time =0 than in their respective supernatants revealing the presence of haemic high-spin Fe (III) chemical systems inside the cells. In fact, immediately after erythrophagocytosis (0h) monocyte pellets showed the typical EPR signal (Table II; Fig. 1B, (ii) spectra). In addition, haemic high-spin Fe (III) appears to be released from monocytes after erythrophagocytosis to the culture medium since 24h after incubation g=6 and g=2 signal is not detected on pellets but only on respective supernatants. According to this findings monocyte after red blood cell phagocytosis appears to oxydise iron to a ferric form maintaining its haemoglobin haemic structure and releasing it latter to the medium.

Iron release as haemoglobin or haemoglobin-like form has been previously described [16,26-28]. While

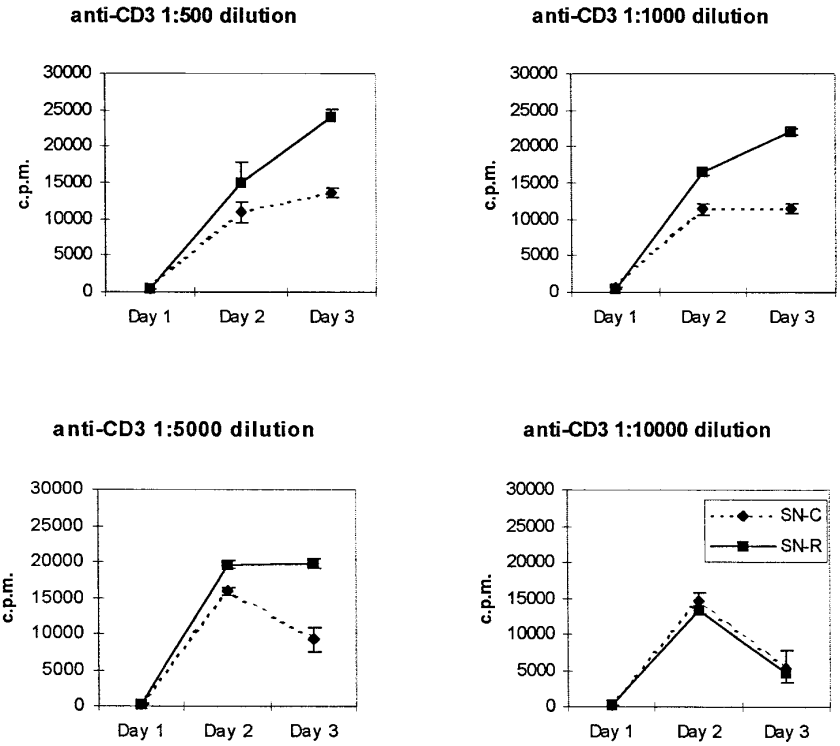

FIG. 5. Anti-CD3 (1:500, 1:1000, 1:5000 and 1:10000 dilutions) activated T cell proliferation obtained in the presence of 12.5% volume (25μl/well) of supernatants from monocyte cultures after erythrophagocytosis (SN-R) and from supernatants monocyte control cultures (SN-C). Results are expressed as average T cell proliferation (cpm)± SD, n=3.

Kondo et al. [16] and Saito et al [27] attributed haemoglobin release to iron toxicity due to a high ingestion of erythrocytes, Moura [26] and Custer [28] suggest that iron release as haemoglobin can be a physiological process. It is possible to consider that iron release in a haemoglobin-like form is a physiological process occurring whenever too many erythrocytes are taken up functioning in this way as a protective mechanism for the cell. The fact that only low concentrations of free or haptoglobin-bound haemoglobin are found in the plasma is not in contradiction with this explanation. In fact, the amount of iron released in a haemoglobin-like form after erythrophagocytosis in humans will be too low to be measured by the standard methods used in the clinical laboratories [26].

In order to determine the possibility of immunomodulatory activity of supernatants from monocyte cultures after erythrophagocytosis, T lymphocyte proliferation assays were performed. The results showed the existence of co-mitogenic properties of supernatants from monocyte cultures that phagocytose RBC.

Haemin has been previously reported as a metalloporphyrin with mitogenic and co-mitogenic properties [10,12-14]. Since haemin is a ferric system with similar chemical characteristics to the iron compounds found in culture supernatants after erythrophagocytosis we suggest our results could be explained by the presence of haemin on these supernatants. Stronger support for this hypothesis is the fact that compounds that are metabolic or structurally related to haemin are neither mitogenic nor co-mitogenic [13]. Protoporphyrin X, which is nonmitogenic, is rendered mitogenic by the chemical insertion of iron to form haemin [13]. In this way, it's not supprising the denomination of "ferro-mitogens" to a variety of iron-containing proteins with lymphocyte stimulatory properties, including haemin [12].

Haemin immunomodulatory effects can also be explained by stimulation of TNF-α release from peripheral MNC [10, 12]. It is also possible that the metalloporphyrins have a direct stimulatory effect on macrophages to produce TNF-α [10]. In fact, results from ELISA. assays showed that TNF-α concentration was increased in supernatants of monocyte cultures after erythrophagocytosis compared to control supernatants. According to these data we suggest TNF-α to be a possible candidate for the co-stimulatory properties of supernatants from monocyte cultures after erythrophagocytosis.

Very interesting is the fact that metalloporphyrin-induced mitogenesis has a stringent requirement for macrophages. In this way, macrophages seem not only providing accessory signals to T cells proliferation but could also mediate at least in part the mitogenic effect of metalloporphyrins [10].

According to the results from this work, the involvement of iron compounds derived from erythrophagocytosis as possible agents of interaction between the immunologic system and the iron metabolism seem clear and add to the numerous reports of the significant functional reciprocal interactions between iron and the immune system [1, 3-8, 26,29-30]. Further studies are still needed however to fully understand the extend of this interaction and its *in vivo* counterpart.

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