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The augmentation of lymphokine-activated killer activity following pulsing of human peripheral blood mononuclear cells with recombinant human interleukin-2

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Summary. The short-term exposure of peripheral blood mononuclear cells (PBMC) to recombinant human interleukin-2 (rhIL-2) at 37°C leads to the generation of lymphokine-activated killer (LAK) activity similar in magnitude to that obtained by the exposure of PBMC to rhIL-2 continuously for 3–5 days. In order to investigate whether the required signal for LAK induction occurred during the short exposure to rhIL-2 or at a later point in the induction phase, PBMC were exposed to rhIL-2 for 1 h at 4°C and then exposed to a low-pH wash to remove bound IL-2 from its receptor. PBMC treated in such a way showed increased LAK activity and proliferation compared to cells exposed to rhIL-2 alone. Expression of the p55 (α) subunit of the IL-2 receptor was also increased. In order to cause the augmentation, a lowering of the pH below 4.0 was necessary, and exposure of PBMC to low pH alone (in the absence of rhIL-2) failed to cause activation. Another relevant feature was a transient increase in the expression of the p75 subunit of the IL-2 receptor (β chain) immediately following the exposure to low pH and the release of interferon γ, tumour necrosis factor α and IL-6; activation was blocked by the inclusion of neutralising antisera raised against rhIL-2 and interferon γ, thus demonstrating that the endogenous release of these cytokines is important for activation.

Key words: Interleukin-2 – LAK cells – Low pH

Introduction

The incubation of peripheral blood mononuclear cells with recombinant human interleukin-2 (rhIL-2) for 3–5 days leads to the generation of cells capable of lysing natural-killer(NK)-cell-resistant human cell lines. This activity has been termed lymphokine-activated killer (LAK) activity [9, 10]. The cells thought to mediate this activity originate from two distinct precursor populations: the majority derive from $CD3^-$, $CD16^+$ cells and the minority from $CD3^+$, $CD16^-$ cells [19, 21]. Protocols using either rhIL-2 alone or rhIL-2 with the reinfusion of syngeneic LAK cells have been used in the treatments of human tumours. The response to this therapy has not reflected the success noted in previous animal studies [16, 17]. Malignant melanoma and renal cell carcinoma have shown the best clinical responses (25% partial or complete responses reported) whilst other types have responded less well [27, 36].

It has recently been demonstrated that short-term exposure of peripheral blood mononuclear cells (PBMC) to rhIL-2 (for between 10 min and 2 h) leads to the generation of LAK activity that is similar in magnitude to that obtained from PBMC exposed to rhIL-2 continously for 4 days [13, 20]. The mechanism of such activation is unclear. It has been suggested that activation, following short-term exposure of PBMC ro rhIL-2, was a direct one, brought about by rhIL-2 binding to its receptor, and was not due to the production of secondary mediators [13]. We have shown that rhIL-2 binding was necessary for activation, and that endogenously produced interleukin-2 was also essential for the generation of cytolytic LAK effector cells [4]. To investigate whether activation by rhIL-2 occurred as a result of signal transduction following a short incubation with rhIL-2 or at a later time, a procedure was used to disassociate rhIL-2 from its receptor. Previous reports have shown that exposure to low pH caused disassociation of antibody-antigen complexes, and exposure to low pH has been used to disassociate this cytokine from its receptor [26]. However, when this procedure was used on PBMC after short-term exposure to rhIL-2, LAK activity and proliferation were enhanced. These results are presented below and possible mechanisms involved are discussed.

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Materials and methods

Isolation of PBMC. Samples of heparinised venous blood were obtained from healthy volunteers. PBMC were isolated on lymphocyte separation medium (Lymphoprep-Nycomed, Norway) as previously described [2]. The recovered lymphocytes were washed three times in cold phosphate-buffered saline (PBS) and resuspended in AIM-V, a serum-free medium (Gibco, Grand Island, USA).

rhIL-2 pulsing. Recombinant human interleukin-2 was a gift from Euro CETUS (Amsterdam, The Netherlands) and units referred to in the text are cetus units (1 cetus unit, U, is equivalent to 6 international units). The specific activity of rhIL-2 supplied was 3.0×10^6µ/mg. PBMC were exposed to rhIL-2 for 1 h at either 37° C or 4° C (procedures at 4° C were performed using precooled equipment and a refrigerated centrifuge). After extensive washing PBMC were adjusted to 4×10^6 cells/ml and recultured for 4 days at 37° C in 5% CO_2. PBMC were harvested by vigorous pipetting and biological assays performed.

Exposure of PBMC to low pH. PBMC pulsed with rhIL-2 were centrifuged and the pellets resuspended in 1 ml AIM-V medium adjusted to pH 3.0 for 1 min. A 20-ml sample of cold PBS was added to the tube and the cells were washed three times. The density of the cells was adjusted to 4×10^6 cells/ml in AIM-V medium and these were placed in 1-ml volumes in 24-well plates (Costar, Cambridge, USA). They were incubated at 37° C in 5% CO_2 for 4 days.

Four-hour ^{51}Cr-release cytotoxicity assay. Cytotoxicity was assessed by means for a 4-h chromium-release assay against the NK-resistant human colon carcinoma cell line, SW742. Briefly, target cells were pelleted and resuspended; 3.7 mBq sodium [^{51}Cr]chromate was added and the mixture was incubated for 1 h at 37° C. The targets were washed once in RPMI medium (centrifuged at 400 *g*). The targets were then resuspended in 10 ml RPMI medium and were incubated for 1 h at 37° C. Following a further two washes in RPMI medium, a viable cell count was performed using 0.1% trypan blue exclusion. Cytotoxicity was assessed in triplicate in Falcon flexible 96-well round-bottom plates (Becton Dickinson). Target cells were used at 10^5 ml (in 0.1-ml volumes) and were incubated with effector cells (0.1 ml) at ratios greater than 2:1 effector: target (E:T). Target cells were also incubated alone in the presence of 0.1 ml RPMI medium. Plates were incubated for 4 h at 37° C in a 5% CO_2/air humidified atmosphere after which 100 µl supernatant was removed into corresponding wells and the plates were dried. Individual wells were counted on a gamma counter and ^{51}Cr release was calculated as follows:

$$^{51}\text{Cr release (\%)} = \frac{(^1/_2\, sn) \times 2}{(^1/_2 sn + p) + {}^1/_2\, sn} \times 100$$

where sn = ^{51}Cr release from the supernatant and, p = ^{51}Cr release from the cell pellet,

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

Measurement of proliferation. Proliferation was assessed by measuring the incorporation of [^{3}H]thymidine (Amersham, UK). Briefly, 10^5 PBMC (in 0.1-ml volumes in 96-well plates) were pulsed for 4 h with 0.5 µCi [^{3}H]thymidine. The cells were harvested on to a filter-paper using an automated cell harvester (Skatron, Norway) and incorporation of [^{3}H]thymidine was measured using a β-spectrophotometer.

Fluorescence-activated cell sorting (FACS) analysis. Expression of p55 (Tac antigen or α chain) and p75 (β chain) subunits of the IL-2 receptor was assessed by flow cytometry. Briefly, 5×10^5 cells/test were incubated with either 100 µl 1/60 diluted mouse anti-human IL-2 α chain receptor (Dakopatts, Denmark) or mouse anti-human IL-2 β chain (Mik-1 Nichirei Corporation, Japan) for 20 min at 4° C. Following washing with cold PBS, 100 µl of a 1/60 diluted goat anti-(mouse IgG) conjugate (Calteg, San Fransisco, USA) was added and incubated for 20 min at 4° C. The cells were washed again and resuspended in 0.4 ml cold PBS; the fluorescence was then assessed on a FACS 420 B.D. (FACS systems, Becton Dickinson, Sunnyvale, Calif.). The results were expressed as the percentage of positive cells with respect to control samples. Mean channel values were measured in arbitrary linear units considered to be proportional to antigen density.

IL-2 binding. IL-2 binding was assessed using rhIL-2 conjugated to phycoerythrin (IL-2-PE). Briefly, 5×10^5 cells/test were incubated for 1 h at 4° C with 10 µl/test rhIL-2–PE (rhIL-2–PE was supplied by British Biotechnology at a concentration of 2.5 µg/ml and had a specific activity of 3.0×10^6 U/mg). After washing, the fluorescence was assessed by flow cytometry at a wavelength of 414 nm.

Cytokine measurements. Supernatants taken from pulsed LAK cell cultures were frozen at –80° C and subsequently assayed for the presence of tumour necrosis factor α (TNFα), interferon γ (IFNγ) and IL-6 using enzyme immunoassay as previously described [15]. Briefly micro-ELISA 96-well plates were coated with 50 µl/ml polyclonal antibody (against the cytokine being assayed) diluted in carbonate buffer (pH 9.6) for 2 h at 37° C in a humidified atmosphere. The antibody was removed and non-specific binding sites were blocked by overnight incubation with 2.5% bovine serum albumin in TRIS-buffered saline at 4° C (150 µl/well). The plates were washed three times in TRIS/saline with 0.1% Tween 20. Cytokine standards, 50 µl/well diluted to an appropriate concentration range, were added in triplicate and test samples were added in duplicate and the plates were incubated for 1 h at 37° C in a humidified atmosphere. After further washing, 50 µl/well mouse monoclonal antibody (against the cytokine being measured) was added and the plate was incubated for a further 1 h at 37° C. After washing, biotinylated sheep anti-mouse IgG (Amersham RPN 1001) was added and incubated for 1 h at 37° C; this was followed (after washing) by streptavidin conjugated to alkaline phosphatase (Amersham RPN 1234). After a further 30-min incubation, 50 µl/well substrate (Sigma 104) was added to 50 µl substrate buffer and the plates were incubated at 37° C until the top standard reached an absorbance (414 nM) of between 1.5 and 2.0. A standard curve was constructed and concentrations of cytokine in the test samples were read off it.

Antisera experiments. In experiments where antisera were used, the cells were prepared as described and the antisera added. The rabbit anti-(human rhIL-2) was prepared as described below and was used routinely at a 1/100 dilution, a dilution known to neutralise the biological activity of 500 U/ml rhIL-2.

Production and purification of polyclonal antisera against rhIL-2. Rabbits were bled and sera collected prior to immunisation with 100 µg rhIL-2 (Glaxo, Switzerland; specific activity = 3.1×10^6 U/mg as measured by [^{3}H]thymidine uptake by CTLL cells) in 0.5 ml complete Fruend's adjuvant (CFA) and sera were collected 10–14 days later. Rabbits were boosted as above without CFA at 3 weeks and sera collected 10–14 days later. To purify the serum, 5 ml rabbit sera was used with both pre-bleed and boost sera purified in parallel. IgG was precipitated using 35% ammonium sulphate overnight at 4° C and the precipitate resuspended in 1 ml PBS followed by dialysis with two changes of 0.1 M phosphate buffer pH 7.2/0.5 ml NaCl. IgG was purified using a Sephacryl S200 HR gel filtration column (column volume 160 ml) and 2-ml fractions were collected. The fractions were analysed for anti-IL-2 activity using enzyme-linked immunosorbent assay (ELISA); peak fractions were pooled and then concentrated in an Amicon Contriprep concentrator and stored in aliquots at –80° C. Neutralising anti-IFNγ was prepared in a similar manner using 750 µg/ml IFNγ in the immunisation. This was also used at a 1/100 dilution, a dilution shown to neutralise in excess of 500 U/ml rhIFNγ.

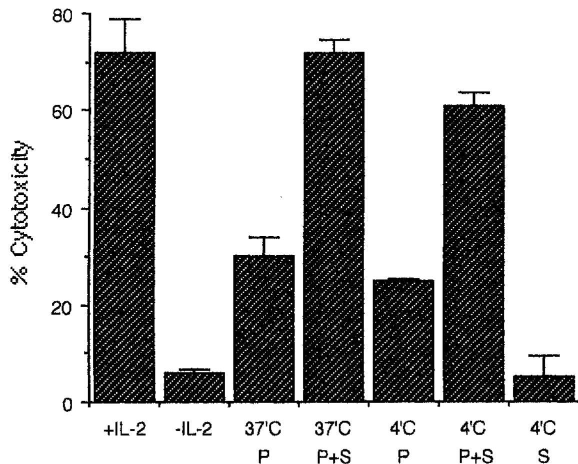

Fig. 1. Cytotoxicity against SW742 by peripheral blood mononuclear cells (PBMC) pulsed with recombinant human interleukin-2 (rhIL-2) and treated at pH 3.0. PBMC were incubated with 500 U/ml rhIL-2 for 1 h, washed once and exposed to AIM-V medium, pH. 3.0, for 1 min at 4° C and 37° C; this was followed by extensive washing in cold phosphate-buffered saline (PBS). Cell numbers were adjusted to 4×10^6 cells/ml and cultured for 4 days at 37° C, 5% CO_2, after which time they were assayed for cytotoxicity against SW742 cells in a 4-h chromium-release assay. Results are representative of ten experiments performed. *P*, pulsed for 1 h with rhIL-2; *S*, exposed to AIM-V medium, pH 3, for 1 min

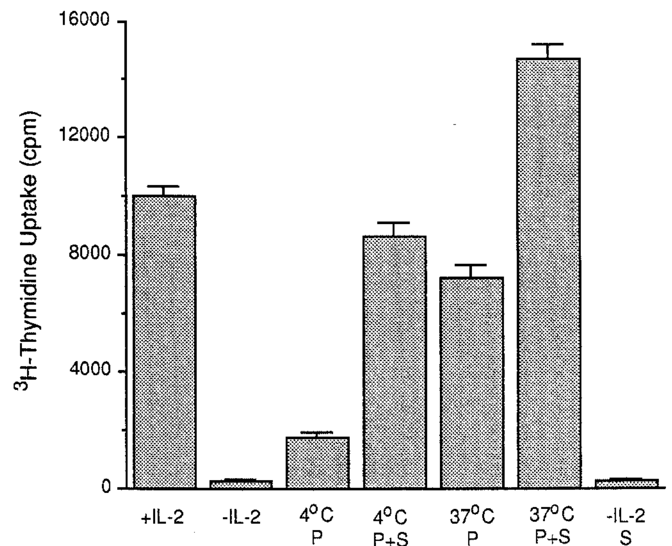

Fig. 2. Proliferation of PBMC following pulsing with rhIL-2 and treatment at pH 3.0. PBMC were cultured for 1 h with rhIL-2 at both 4° C and 37° C, exposed to low pH for 1 min and then washed extensively in cold PBS. The cells were adjusted to a cell density of 4×10^6 cells/ml in AIM-V medium and were cultured at 37° C for 4 days, after which time proliferation was assessed by a tritiated thymidine uptake assay. Results are representative of eight experiments. *P*, pulsed for 1 h with 500 U/ml rhIL-2; *S*, exposed to AIM-V, pH 3.0, for 1 min

Results

PBMC activation following rhIL-2 pulsing and exposure to low pH

PBMC were pulsed with rhIL-2 at both 4° C and 37° C and exposed to AIM-V medium, pH 3.0, for 1 min prior to extensive washing in cold PBS. After 4 days in culture in the absence of exogenous cytokines, cytotoxicity was assessed in a 4-h chromium-release assay against SW742.

The exposure to low pH after rhIL-2 pulsing caused an increase in cytotoxic activity compared to PBMC pulsed with rhIL-2 alone (Fig. 1). The level of cytotoxicity generated was similar to that obtained with PBMC cultured in AIM-V medium containing 500 U/ml rhIL-2 for 4 days. PBMC exposed to low pH without rhIL-2 pulsing showed no increase in cytotoxicity. In addition, treatment of PBMC with low pH prior to exposure to rhIL-2 did not cause increased cell activation (data not shown). When the pulsing and exposure to low pH were performed at 4° C, an increase in cytotoxicity above that achieved by rhIL-2 pulsing alone was observed in 10/10 experiments; this increase was significant as assessed by the Wilcoxon signed-rank test $P < 0.001$. Similarly, when the activation procedure was performed at 37° C the cytotoxic response to pulsing and exposure to low pH was increased in 9/12 experiments, which was not significant. The proliferation of PBMC exposed to rhIL-2 and low pH was assessed on day 4 by a [^{3}H]thymidine uptake assay. The results showed an increase in the proliferation rate of PBMC exposed to rhIL-2 and low pH compared to the values obtained from PBMC exposed to rhIL-2 alone (Fig. 2), at both 4° C (10/10 experiments) and at 37° C (8/10 experiments). The difference in proliferation was statistically significant at both 4° C $P < 0.001$ and 37° C $P < 0.05$.

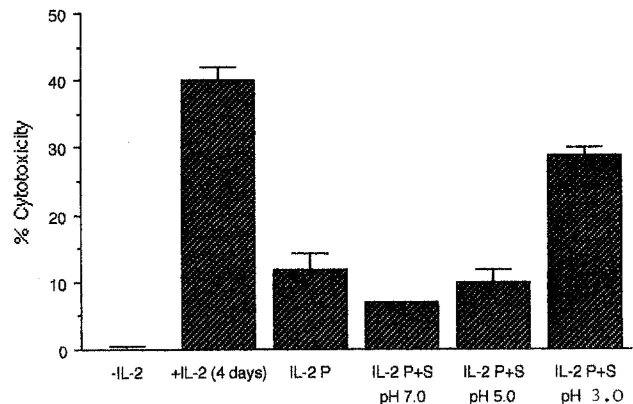

Fig. 3. PBMC activation following rhIL-2 pulsing and low-pH treatment. Cytotoxicity against SW742 cells of PBMC cultured for 1 h with rhIL-2 (500 U/ml) at 4° C then exposed to AIM-V medium at pH 7, 5 and 3 for 1 min. After extensive washing in cold PBS, the cells were incubated for 4 days at 37° C in 5% CO_2 and were assessed for cytotoxicity by means of a chromium-release assay. Results are representative of eight experiments. *P*, pulsed for 1 h with rhIL-2; *S*, exposed to AIM-V at pH 3, 5, or 7

To determine the pH required for augmentation, PBMC were pulsed with 500 U/ml rhIL-2 for 1 h and exposed to AIM-V medium adjusted to different pH values (Fig. 3). Medium at pH 5.0 had no effect on the cytotoxicity generated by rhIL-2 pulsing; however, exposure of PBMC to a pH below pH 5.0 augmented the cytotoxicity compared with that of pulsed control PBMC; maximum activation occurred at pH 3.0. Similar results were obtained for cell proliferation (results not given). The optimum time for exposure of PBMC to pH 3.0 was shown to be 1 min. An increase in cytotoxicity occurred after a 15-s exposure to medium at pH 3.0; this reached a maximum at 1 min expo-

Table 1. Staining of fresh peripheral blood mononuclear cells (PBMC) and phytohaemagylutinin (PHA)-stimulated PBMC with recombinant human interleukin-2 (rhIL-2) linked to phycoerythrin (PE)[a]

Stain	Fresh PBMC[a]		PHA-stimulated PBMC[a]	
	+ve (%)	Mean channel values[b]	+ve (%)	Mean channel values[b]
Control	3	55	8	94
Tac	8	46	90	334
rhIL-2–PE	40	108	86	488
rhIL-2–PE + unlabelled rhIL-2	22	51	17	44
rhIL-2–PE + low-pH exposure[c]	36	66	82	128
rhIL-2–PE + low-pH exposure[d]	35	59	80	169

[a] PBMC were incubated with rhIL-2–PE for 1 h at 4° C. After washing, cells were exposed to AIM-V medium, pH 3.0, before washing. The rhIL-2 binding was assessed by flow cyometry. Representative of four experiments

[b] Arbitrary linear units

[c] Exposed to glycine/HCl, pH 2.5, for 1 min

[d] Exposed to AIM-V medium, pH 3.0, for 1 min

Table 2. Release of cytokines into tissue-culture supernatant by PBMC either pulsed with rhIL-2 or pulsed with rhIL-2 and exposed to low pH at both 37° C and 4° C followed by a 4-day induction phase[a]

Cell treatment	Release of cytokines (U/ml)			Cytotoxicity against SW742 (%)
	IFNγ	TNFα	IL-6	
With rhIL-2 for 4 days	450	750	1000	60
Unstimulated	25	5	5	0
Pulsed rhIL-2 at 37° C	160	530	60	51
Pulsed rhIL-2 and low pH 37° C	200	510	80	53
Pulsed rhIL-2 at 4° C	30	5	75	15
Pulsed rhIL-2 and low pH at 4° C	160	300	100	42

[a] The levels of interferon γ (IFNγ), tumour necrosis factor α (TNFα) and IL-6 were measured, by enzyme immunoassay from supernatant taken from PBMC pulsed with rhIL-2 with and without subsequent exposure to low pH at both 4° C and 37° C. Representative of three experiments. Relevant cytotoxicity results are included

sure time, a level that was maintained if PBMC were exposed to pH 3.0 for 2 min (results not given). No decrease in PBMC viability was observed when cells were treated at low pH for up to 2 min (as assessed by trypan blue exclusion). In all subsequent experiments a 1-min exposure to AIM-V medium at pH 3.0 was used. Also, pulsing with different concentrations of rhIL-2 (range 500–2000 U/ml) followed by pH 3.0 treatment caused an increase in cell activation compared to the results of rhIL-2 pulsing alone (results not given).

Influence of pH 3.0 treatment on rhIL-2 binding

The effect of low pH on bound rhIL-2 is shown in Table 1. Phycoerythrin(PE)-conjugated rhIL-2 was allowed to bind to both fresh PBMC, and PBMC stimulated for 3 days with phytohaemagglutinin (5 μg/ml). With both activated and non-activated PBMC the binding of rhIL-2–PE was reduced in the presence of unlabelled rhIL-2 (both the percentage of positive cells and mean channel values representing the degree of binding per cell). PBMC exposed to low-pH treatment with either glycine/HCl (pH 2.5) or AIM-V medium (pH 3.0) showed a reduction in the intensity of rhIL-2–PE staining, but not in the percentage of cells stained; results were similar for PHA-activated and non-activated cells.

Cytokine assessment

The importance of released cytokines in the response of PBMC to pulsing with and without exposure to low pH was assessed by measuring the cytokines in the tissue-culture supernatant from PBMC following activation in vitro. This reflected both the release and consumption of cytokines by PBMC. The cytokines measured were TNFα, IFNγ and IL-6, all of which have been reported to up-regulate LAK activation. The levels of cytokines detected in the culture supernatants were compared to the cytotoxic activity of the cells.

In comparison with PBMC exposed to rhIL-2 continuously for 4 days, lower concentrations of all three cytokines were produced by PBMC pulsed with rhIL-2 (at both 37° C and 4° C) regardless of whether they were exposed to the low-pH wash (Table 2). The results suggested that pulsing followed by low-pH exposure performed at 4° C caused an increased release of both IFNγ and TNFα compared to PBMC that were pulsed with rhIL-2 alone. The release of IL-6 was less markedly affected by the low-pH exposure. There was a less obvious enhancement of cytokine release by exposure of PBMC to low pH at 37° C. Control PBMC cultures in the absence of rhIL-2 released only low levels of all three cytokines.

The cytotoxicity of activated PBMC was greatest when IFNγ release was highest. When analysed over a 96-h induction phase this trend was confirmed, suggesting that IFNγ may be an important mediator of the cytotoxic response following pulsing and exposure of PBMC to low pH (data not shown).

In order to investigate further the role of IFNγ in PBMC activation, anti-IFNγ polyclonal neutralising antibody was added to PBMC after pulsing and pulsing plus low-pH exposure. This led to decreased LAK induction regardless of the method of rhIL-2 activation (Fig. 4) in 4/4 experiments. The inclusion of control serum (obtained from the rabbit prior to immunisation) had no effect on LAK induction. It is also important to note that the inclusion of neutralising antisera against IFNγ had no effect on the proliferative response of PBMC following activation by rhIL-2 (results not shown).

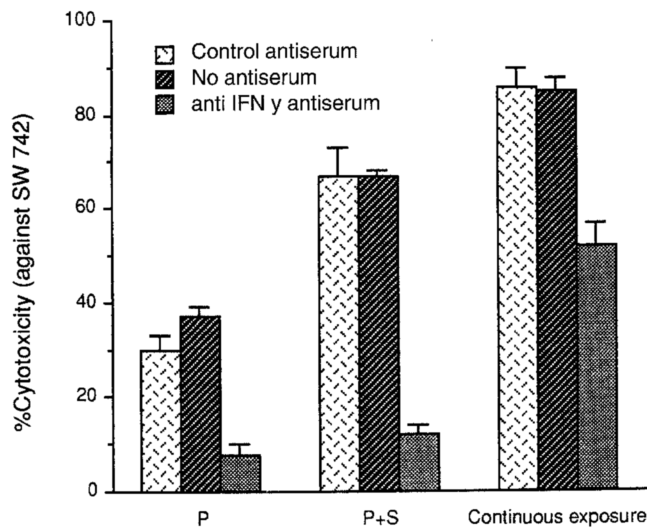

Fig. 4. The effect of the addition of anti-interferon-γ (IFNγ) on PBMC pulsed with rhIL-2 or pulsed and exposed to low pH. PBMC were pulsed with rhIL-2 (at 4° C), either with or without exposure to low pH, and were cultured at 37° C for 4 days in the presence of neutralising anti-IFNγ. Cytotoxicity was assessed by means of a 4-h [51]Chromium-release assay. *P*, pulsed for 1 h with 500 U/ml rhIL-2; *S*, exposed to AIM-V, pH 3.0 for 1 min

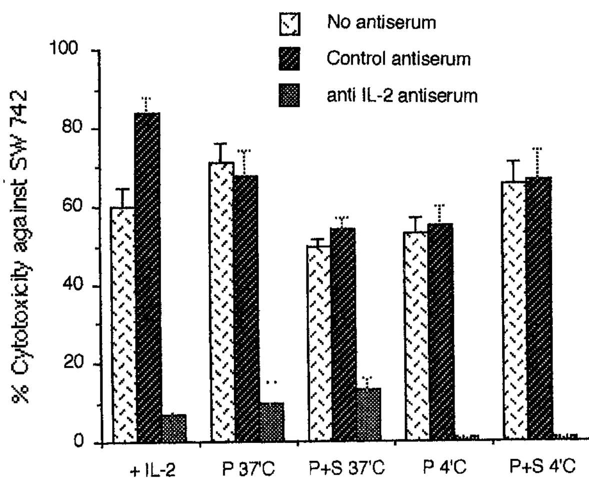

Fig. 5. Effect of the addition of anti-rhIL-2 on PBMC pulsed with rhIL-2 or pulsed and exposed to low pH. PBMC were pulsed with rhIL-2 (at either 37° C or 4° C), exposed to low pH and then cultured at 37° C for 4 days in the presence of neutralising anti-rhIL-2 serum. Cytotoxicity was assessed by means of a 4-h chromium-release assay. *P*, pulsed for 1 h with rhIL-2; *S*, exposed to AIM-V, pH 3.0, for 1 min

Role of endogenous IL-2 production

In order to study the role of endogenously produced IL-2 in PBMC activation, neutralising polyclonal anti-rhIL-2 was added to PBMC during the induction phase, following pulsing with rhIL-2 and low-pH treatment at both 37° C and 4° C (Fig. 5). The addition of antisera had an inhibitory effect on PBMC pulsed with rhIL-2 or pulsed and subsequently treated at pH 3.0 (4° C or 37° C). Inclusion of control sera (sera obtained prior to immunization) had no effect on PBMC activation.

Table 3. Expression of p55 (α) and p75 (β) subunits of the IL-2 receptor on PBMC following rhIL-2 pulsing or rhIL-2 pulsing and exposure to low pH[a]

Cell treatment	Immediate analysis (% cells positive)		4-day analysis (% cells positive)	
	p55	p75	p55	p75
Untreated	2.75 (28)	8.69 (29)	9 (35)	6.93 (39)
rhIL-2 pulsed	1.29 (35)	2.6 (27)	29.2 (72)	6.9 (41)
rhIL-2 pulsed and low pH	2.18 (39)	22.44 (26)	37.03 (99)	5.18 (54)
rhIL-2 present for 4 days	NT	NT	22.14 (70)	12.12 (32)

[a] PBMC were pulsed with rhIL-2 with or without subsequent exposure to low pH. They were analysed immediately and after 4 days for expression of p55 and p75 subunits of the IL-2 receptor by flow cytometry. Results are representative of four experiments. NT, not tested; numbers in parentheses represent mean channel values (arbitrary linear units)

IL-2 receptor subunit expression

The effect of low pH on the expression of the α and β subunits of the IL-2 receptor on PBMC was examined by flow cytometry (Table 3). PBMC were pulsed at 4° C with and without low pH treatment, and the cells stained immediately with monoclonal antibodies against the p55 and p75 subunits of the IL-2 receptor. The percentage of cells staining positive for the p55 (α) subunit was unaffected by low-pH treatment and this observed only at low intensities on fresh PBMC (Table 3). The p75 (β) receptor subunit was expressed at low levels on untreated PBMC and on PBMC pulsed with rhIL-2; however, the percentage of cells expressing the IL-2 β chain increased on cells after exposure to low pH for 1 min. This was evident on PBMC pulsed with rhIL-2 before low-pH treatment and on cells subjected to low pH without prior rhIL-2 pulsing. Scatter analysis of these cells suggested that the expression of p75 was mainly on the monocyte population. After 4 days in culture the expression of p55 subunit increased on PBMC stimulated with rhIL-2, but not on unstimulated PBMC (Table 3). The expression of the p55 subunit was increased on cells pulsed and exposed to pH 3.0 compared with cells pulsed alone.

Discussion

The short-term exposure of PBMC to rhIL-2 for between 10 min and 2 h at 37° C led to the generation of LAK activity that was similar in magnitude to that obtained from PBMC exposed continuously to rhIL-2 for 4 days. In the present study PBMC were activated by short-term exposure to rhIL-2 for 1 h at 4° C. The advantage of this procedure was a greater cell recovery. The cytotoxicity generated was, however, lower than that obtained from PBMC exposed to rhIL-2 for 1 h at 37° C or PBMC exposed continuously to rhIL-2 for 4 days. The mechanisms of activation at 4° C were thought to be similar to those in operation at 37° C; rhIL-2 was bound to its receptor at 4° C

and at this temperature no internalisation would occur [7]; however, when the cells were removed to 37° C, rhIL-2 would rapidly be internalised.

In order to investigate whether the generation of LAK activity following rhIL-2 exposure at 4° C was due to bound rhIL-2 being internalised during the culture period at 37° C, a series of experiments were performed using a low-pH wash to remove bound ligand from its receptor. Similar protocols have previously been used to remove rhIL-2 from its receptor [7, 13] and also in other ligand/receptor systems [23, 29, 31]. Studies with phycoerythrin linked to rhIL-2 showed that most, but not all the rhIL-2 was removed from PBMC following exposure to low pH. Flow-cytometry studies also showed no difference in the expression of CD3 or CD16 antigen by PBMC exposed to rhIL-2 alone or to rhIL-2 and low pH, suggesting that the augmented IL-2 response was not due to the preferential expression on T cells or NK cells. At 4° C, exposure to low pH led to increased cytotoxicity and proliferation. At 37° C, although there was a similar trend observed, this was not statistically significant. It should, however, be noted that at 37° C some cytotoxicity values from cells exposed to rhIL-2 alone (for 1 h) were very high and subsequent exposure to low pH did not lead to an increase. A possible explanation is that PBMC were maximally stimulated by exposure to rhIL-2 alone; this may reflect the speed of the rhIL-2 signal transduction at this temperature. In addition, at 37° C, internalisation of rhIL-2 may have occurred.

There were a number of important features associated with low-pH activation. Activation was dependent on prior exposure of PBMC to rhIL-2; exposure of PBMC to low pH alone led to no activation. Furthermore, the exposure of PBMC to low pH prior to rhIL-2 did not lead to any increased cytotoxicity compared with levels reached by PBMC exposed to rhIL-2 alone. Results obtained using a number of different low-pH media confirmed that low pH is the important factor as did the results titrating the pH of the media.

The mechanism by which the exposure of PBMC to low pH after rhIL-2 exposure leads to activation was not clear. One possibly important consequence of the low-pH treatment was the rapid but transient increase in the expression of the p75 subunit of the IL-2 receptor. The levels of the p55 (α) subunit of the IL-2 receptor on fresh PBMC were very low and precluded any role in IL-2 binding. This agrees with published results on IL-2α receptor expression on fresh PBMC [18]. With the availability of monoclonal antibodies binding specifically to the β chain of the IL-2 receptor [32, 33], p75 (β) subunit expression was examined. From previous studies, it was clear that, unlike the α chain, the IL-2β receptor was expressed constitutively on many cell types [6, 18, 22] and was primarily responsible both for signal transduction [1] and internalisation of bound rhIL-2 [25]. The data presented here agree with published results; phycoerythrin-conjugated rhIL-2 bound to fresh PBMC, and the expression of the subunit of the IL-2 receptor was also observed on fresh PBMC albeit at low levels. Any effect of low pH on the bound rhIL-2 must have occurred via the p75 receptor, with the possible involvement of a tyrosine kinase that has been recently associated with the β chain [8]. This represents a possible point whereby transduction of the rhIL-2 signal could be augmented, and merits further investigation. It should be noted that the increased expression of the p75 subunit on PBMC does not fully account for the increased activation, since cells exposed to low pH alone showed no cytotoxicity or proliferation above background, although the expression of p75 was raised. The biological significance of the transient increase in the p75 subunit expression on monocytes is unclear. rhIL-2 has, however, been shown to act on monocytes, causing increased hydrogen peroxide activity [12] and increased production of TNFα and IL-1 mRNA [30, 11] as well as enhancing their tumoricidal and antimicrobial activity [34, 24]. Furthermore, monocyte-depleted populations showed no decrease in LAK induction following rhIL-2 pulsing and this response could be augmented by exposure to low pH. Despite these observations, it is unlikely that monocytes positively influence LAK induction following exposure of PBMC to rhIL-2. A further possibility is that other cytokines were also involved in cell activation, mediating the induction of LAK activity. The down-regulating effects of neutralising anti-rhIL-2 sera and anti-IFNγ suggested that endogenously produced IL-2 and IFNγ were important in the induction of LAK activity following both short-term exposure to rhIL-2 and exposure to low pH. Endogenously produced IL-2 may, however, have an individual role in the cytotoxicity generated from PBMC exposed to rhIL-2 and low pH. A possible role for IFNγ was also suggested, although other studies have failed to confirm IFNγ as a cytokine important in activating NK cells and inducing LAK activity [5, 14, 28, 35].

Furthermore, IFNγ has also been demonstrated to overcome growth factor-β and IL-4-mediated inhibition of rhIL-2-induced LAK cytotoxicity [3]. We have shown that IFNγ is released specifically from PBMC exposed to short-term rhIL-2 and to low pH at 4° C, and this may have a role in increasing PBMC cytotoxicity. The results presented here were obtained using a mixed cell culture system, and further studies would be necessary using an enriched purified population of cells in order to determine the contribution of the different subpopulations. In conclusion, we have demonstrated that short-term exposure of PBMC to rhIL-2 followed by exposure to low pH and reculture led to increases in cytotoxicity, proliferation and Tac (IL-2α chain) expression compared to those obtained from PBMC pulsed with IL-2 alone. Whilst the exact mechanisms are still unclear, the increase in the IL-2β chain (p75) together with the increased release of endogenously produced IL-2 and IFNγ may all contribute in this unique activation pathway.

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