Immunology 1995 **84** 135–141

Rat, mouse and human neutrophils stimulated by a variety of activating agents produce much less nitrite than rodent macrophages

E. L. PADGETT* & S. B. PRUETT *Department of Biological Sciences, Mississippi State University, Mississippi, USA*

SUMMARY

The role of reactive nitrogen intermediates (RNI) in the antimicrobial activities of neutrophils from various mammalian species is unclear. However, it has been reported that rodent neutrophils possess the inducible form of nitric oxide synthase and that inflammatory neutrophils from rats produce potentially antimicrobial levels of RNI. In the present study, neutrophils from humans, rats and mice were evaluated for production of nitrite, a stable end-product of RNI. Human neutrophil preparations (>95% neutrophils) isolated from peripheral blood were stimulated for 2–24 hr with agents known to trigger the Ca^{2+}-dependent constitutive nitric oxide synthase, or to stimulate synthesis of the inducible nitric oxide synthase. Superoxide dismutase was added to some cultures to decrease the levels of superoxide, a compound reported to react with RNI and yield products other than nitrite. Even though the cells were viable and responsive to stimuli, they did not produce nitrite concentrations indicative of antimicrobial potential. Preparations of inflammatory (casein-elicited) mouse neutrophils also failed to produce high concentrations of nitrite. Inflammatory rat neutrophils ($2{\cdot}5 \times 10^6$/ml) produced nitrite concentrations of $\sim 40\ \mu$M in 24-hr cultures, but plots of nitrite production versus cell number for neutrophil and macrophage preparations indicated that contaminating macrophages could account for all the nitrite production in the neutrophil preparations. Thus, neutrophils from rats, mice and humans seem comparable in their inability to produce high levels of nitrite in response to a variety of stimuli. This suggests that in most circumstances the constitutive nitric oxide synthase known to be present in these cells is limited to the production of low levels of nitric oxide for intercellular signalling. In addition, this raises questions about the presence or functional status of inducible nitric oxide synthase in rodent neutrophils.

INTRODUCTION

The role of reactive nitrogen intermediates (RNI) in the antimicrobial activities of mammalian neutrophils is uncertain. RNI seem to play a role in the antimicrobial and anti-tumour functions of rodent macrophages,[1–5] and it has been reported that RNI are produced by rat inflammatory neutrophils in sufficient quantities to suggest possible antimicrobial activity.[6] It has been suggested that inflammatory neutrophils from rats and mice possess the inducible form of nitric oxide synthase (iNOS), which is capable of producing high concentrations of RNI.[7] However, the evidence for this assertion is very limited and does not clearly establish that neutrophils are capable of production of RNI at levels sufficient to provide antimicrobial or anti-tumour activity.

Even if it was clear that rodent neutrophils produce antimicrobial levels of RNI, this would not necessarily indicate that this is also true of human neutrophils. Rodent macrophages produce >10 times more nitrite than human macrophages cultured under similar conditions,[8,9] and it is possible that such species differences also apply to neutrophils. The concentration of nitrite, a stable breakdown product of RNI, produced by human neutrophils suggests little if any RNI production.[10] However, there is evidence that human neutrophils produce sufficient concentrations of nitric oxide to function pharmacologically as neutrophil-derived relaxing factor.[11] Such low-level, short-term production of RNI is generally mediated by the constitutive, Ca^{2+}-dependent nitric oxide synthase (cNOS).[7] Although it is generally assumed that this enzyme does not produce sufficient quantities or RNI to permit antimicrobial action, there has been little effort to induce RNI production by neutrophils optimally in order to compare their capabilities in this regard to those of macrophages. A report that cytoplasts prepared from human neutrophils kill staphylococci by an RNI-dependent mechanism suggests that these cells can produce antimicrobial levels of RNI.[12]

These matters must be resolved in order to understand the

Received 13 April 1994; revised 1 September 1994; accepted 2 September 1994.

* Present address: Department of Cellular Biology and Anatomy, LSU School of Medicine, Shreveport, LA, USA.

Correspondence: Dr S. B. Pruett, Department of Biological Sciences, PO Drawer GY, Mississippi State, MS 39762, USA.

relative importance of RNI and other antimicrobial mechanisms in rodent neutrophils, and to assess the relevance of rodent neutrophils as a model for human neutrophils.

The present study was conducted to compare the ability of neutrophils from rats, mice and humans to produce nitrite at levels consistent with antimicrobial or anti-tumour activity. A variety of soluble and particulate stimuli was used in an attempt to optimally activate neutrophils for RNI production. Rodent macrophages were included in some experiments as positive controls. The results suggest inflammatory neutrophils from rats and mice do not produce sufficient concentrations of nitrite to indicate an important role for neutrophil-derived RNI as antimicrobial or anti-tumour agents. This was also true for human neutrophil preparations. It remains possible that there are unique conditions or combinations of stimuli *in vivo* that could induce such activity, but it is clear that neutrophils and macrophages differ with regard to the stimuli required to induce or trigger high-level RNI production.

MATERIALS AND METHODS

Animals

Female B6C3F1 mice were obtained through the National Cancer Institute's (Bethesda, MD) animal programme. The mice were 6–8 weeks old on arrival, and were given at least 2 weeks to recover from shipping stress and to become acclimatized to the new environment prior to use in experiments. Mice were housed in an American Association for the Accreditation of Laboratory Animal Care (AAALAC) accredited facility with a 12-hr light/dark cycle and were given food (Purina lab chow) and water *ad libitum*. Sentinel mice housed in the same room were negative for mouse hepatitis virus, Sendai virus and *Mycoplasma pulmonis* during these experiments. Male Fisher 344 rats were obtained from Charles River Breeding Labs (Wilmington, MA) and were housed in a different room in the same animal facility. They were allowed to recover from shipping stress and to acclimatize for at least 3 weeks prior to use at 12–24 weeks of age. Animal care and use was in accord with National Institutes of Health (Bethesda, MD) guidelines.

Human neutrophils

Blood was obtained from healthy male volunteers aged 22–38 years by venepuncture into EDTA vacutainers. Neutrophils were isolated by centrifugation over a discontinuous Ficoll gradient, as described previously.[8] Differential cell counts were done using Wright-stained smears.

Mouse and rat macrophages and neutrophils

Macrophage preparations were obtained 78 hr after intraperitoneal injection of a 10% sterile casein solution (in bicarbonate buffer) (1 ml for mice; 3 ml for rats). Neutrophil preparations were harvested 8 hr after the same eliciting stimulus. Cells were harvested by peritoneal lavage after killing by CO_2 inhalation. The lavage fluid (8 ml/animal) was ice-cold RPMI-1640 with 10% fetal bovine serum, 2 mM L-glutamine, 10 U/ml penicillin and 100 μg/ml streptomycin, at pH 6·8 (complete medium). Peritoneal exudates from groups of five to eight mice or two to three rats were pooled, centrifuged at 300 *g*, and resuspended to the appropriate cell density in complete medium. Differential cell counts were done using Wright-stained smears, and absolute leucocyte counts were done using a Coulter counter (Hialeah, FL). Macrophage and neutrophil numbers shown in the Results section were obtained by multiplying the total cell number by the fraction of cells of each type, as indicated by the differential cell count for each cell preparation.

Activation of macrophages and neutrophils

Cell suspensions in complete culture medium were placed into 96-well flat-bottomed cell culture plates. Cells were incubated for either 2 or 24 hr after addition of activating agents, and 100 μl of culture supernatant was removed for measurement of the nitrite concentration. The metabolic activity of the cells was then assessed using the MTT assay (described below). In some of the experiments involving mouse macrophages, the activating agents were removed by three washes in culture medium, fresh culture medium was added, and nitrite concentration was assessed 2 hr later.

Stock solutions of activating agents or dilutions of the stock solutions in complete culture medium were prepared so that a volume of 10 μl added to each well provided the desired concentration. Control cultures received the same amount of complete culture medium. Additional controls received an amount of dimethylsulphoxide (DMSO) comparable to the highest concentration included as a vehicle for an activating agent. The greatest DMSO concentration used was 0·1%, and this had no effect on nitrite production.

Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Sigma Chemical Co., St Louis, MO) was prepared as a stock solution at 100 μg/ml in complete culture medium and stored frozen until needed. Prior to use the LPS stock solution was thawed and extensively vortexed (10 min) to prevent loss by adherence to the container. Human interferon-γ (IFN-γ) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), and mouse IFN-γ was kindly provided by Genentech (San Francisco, CA). Zymosan A (Sigma Chemical Co.) was opsonized (OpZy) by incubation for 45 min at 37° with 20% normal human serum. Heat-killed *Streptococcus* group B (SGB) was prepared by heating log-phase cultures of *Streptococcus* group B (strain 090 from Dr W. Wennerstrom) for 1 hr at 80°. The bacteria were then washed twice by centrifugation and suspended in phosphate-buffered saline (pH 7·2). Platelet-activating factor (PAF), *N*-formyl-methionine-leucine-phenylalanine (FMLP) and phorbol 12-myristate acetate (PMA) were obtained from Sigma Chemical Co., and concentrated stock solutions (1 mg/ml) were prepared in DMSO. These solutions were diluted in complete culture medium prior to addition to cultures. Superoxide dismutase (SOD; Sigma Chemical Co.) was added to some cultures to decrease the concentration of superoxide anion.

Nitrite assay

A 100-μl sample of culture supernatant fluid was removed from each well, and the nitrite concentration was determined using the Greiss reagent, as described previously.[8]

MTT assay

The reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to a purple formazan with an absorbance maximum near 610 nm was used to measure the viability/metabolic activity of cultured cells. The procedure was done for all cell types, as described previously for human neutrophils.[13]

Table 1. Nitrite production by human neutrophils or mouse macrophages in response to various stimuli

Exp.	Species	Cell type	Cell density (cells/ml)	Culture duration (hr)	Ativating agent/SOD*	Nitrite conc. (μM)†	Absorbance at 610 nm (MTT assay)
1	Human	PMN	$1{\cdot}5 \times 10^6$	2	—	ND	0·12 ± 0·01
					—/SOD	0·95 ± 0·00	0·02 ± 0·00
					SGB	ND	0·13 ± 0·01
					SGB/SOD	2·40 ± 0·29	0·04 ± 0·00
					OpZy	ND	0·24 ± 0·00
					OpZy/SOD	0·95 ± 0·00	0·11 ± 0·02
					PMA	ND	0·36 ± 0·00
					PMA/SOD	0·95 ± 0·00	0·18 ± 0·00
		PMN	$1{\cdot}5 \times 10^6$	24	—/SOD	2·01 ± 0·16	ND
					SGB/SOD	1·13 ± 0·23	ND
					OpZy/SOD	0·53 ± 0·00	ND
					PMA/SOD	0·53 ± 0·00	ND
2	Human	PMN	$1{\cdot}5 \times 10^6$	2	—	0·72 ± 0·27	0·22 ± 0·06
					—/SOD	0·89 ± 0·31	0·10 ± 0·03
					LPS	0·64 ± 0·13	0·30 ± 0·04
					LPS/SOD	0·64 ± 0·19	0·16 ± 0·04
					PAF	0·53 ± 0·00	0·19 ± 0·04
					PAF/SOD	0·64 ± 0·19	0·10 ± 0·03
					FMLP/SOD	0·61 ± 0·14	0·13 ± 0·02
3	Human	PMN	$1{\cdot}5 \times 10^6$	24	—	2·37 ± 1·57	ND
					LPS	2·19 ± 1·02	ND
					IFN 500	1·81 ± 1·21	ND
4	Human	PMN	$1{\cdot}5 \times 10^6$	24	—	Undetectable	ND
					LPS/SGB‡	Undetectable	ND
					IFN/SGB	Undetectable	ND
					IFN/LPS/SGB	Undetectable	ND
5–7	Mouse	MØ	$1{\cdot}5 \times 10^6$	2	—	0·72 ± 0·35	ND
					IFN 20	9·05 ± 3·04	ND
					IFN 500	13·43 ± 3·07	ND
					IFN 20/LPS	24·33 ± 6·15	ND
					IFN 500/LPS	24·74 ± 5·77	ND

* Concentrations of activating agents were as follows: SGB, $1{\cdot}5 \times 10^6$ heat-killed bacteria/well; OpZy, 0·1 mg/ml; PMA, 200 nM; LPS exps 1–4, 1 μg/ml; LPS exps 5–7, 10 ng/ml; PAF, 1 μM; FMLP, 1 μM; IFN 500, IFN-γ at 500 U/ml; IFN 20, IFN-γ at 20 U/ml (human IFN-γ was used in exps 3–4 and mouse IFN-γ was used in exps 5–7). SOD was used at 450 U/ml.

† Values shown are means ± SD for triplicate samples, except for exps 5–7 in which the data represent means ± SE for three independent experiments.

‡ In exp. 4, neutrophils were incubated for 24 hr with the indicated stimuli, then SGB was added and the nitrite concentration determined 3 hr later.

ND, not determined.

Statistical analysis

Analysis of variance followed by Bonferroni's *post hoc* test was performed to compare the mean of control cultures to the means of activated cultures. P values $\leqslant 0{\cdot}05$ were regarded as significant.

RESULTS

Nitrite production by human neutrophils

Data listed in Table 1 indicate little if any nitrite accumulation in 2- or 24-hr cultures of human neutrophils exposed to a variety of stimuli. The average purity of the human neutrophil preparations used in this study was 96 ± 2%. Neutrophils for each of the three experiments noted in Table 1 were obtained from a different donor, and neutrophils from two additional donors yielded similar results in several experiments (data not shown).

Stimuli known to induce iNOS expression in macrophages (IFN-γ and LPS), to activate neutrophil functions through a variety of second messenger pathways (OpZy, SGB, PMA and PAF), and to activate cNOS (FMLP)[11,14] were included in the present study. None of these agents induced nitrite concentrations greater than 2·40 μM in 2- or 24-hr cultures. Nitrite concentrations also did not reach detectable levels in cultures treated with various activators then triggered with a particulate stimulus (SGB) (Table 1, exp. 4).

As a positive control, mouse macrophages treated with IFN-γ or IFN-γ plus LPS were evaluated under the same conditions (Table 1). In 2-hr cultures nitrite levels as great as 24·7 μM were observed. Higher levels were noted in 24-hr cultures (data not shown). It should be noted that these

macrophages had been preactivated for 24 hr with IFN-γ and LPS to fully induce iNOS. The cells were then washed prior to initiation of cultures for assessment of nitrite levels. This was done to demonstrate the capabilities of macrophage iNOS-mediated nitrite production during 2- and 24-hr cultures. Because the metabolic activity of activated human neutrophils decreases markedly after 24 hr in culture,[13] this preinduction period was not attempted for neutrophils. Rather, the cells were stimulated with agents known to activate cNOS as well as agents known to induce macrophage iNOS, and nitrite concentrations were measured at times designed to assess rapid nitrite production by cNOS (2 hr) or more gradual nitrite accumulation due to iNOS induction (24 hr). Human neutrophils did not produce nitrite levels at either time-point comparable to those produced by IFN-γ- and LPS-induced mouse macrophages.

It has been suggested that superoxide produced by neutrophils may react with RNI to produce end-products other than nitrite.[11,14] McCall *et al.* demonstrated that addition of superoxide dismutase to cultures of human neutrophils increased the quantity nitric oxide, as detected by a functional assay (platelet aggregation).[14] Therefore, low nitrite concentrations in human neutrophil cultures in the present study might have been caused by this reaction, and not by lack of RNI production. To evaluate this possibility, superoxide dismutase (SOD) was used in two experiments to decrease superoxide concentrations. Cultures treated with SOD did not contain significantly higher concentrations of nitrite than matching cultures without SOD (as assessed by ANOVA followed by Bonferroni's test).

The viability of the human neutrophils used in this study, the effectiveness of the activating stimuli, and the activity of SOD were assessed by spectrophotometric measurement of the reduction of MTT in neutrophil cultures following 2 hr of exposure to activating stimuli (Table 1). This method is widely used to measure viability of a number of cell types, including neutrophils.[13,15] Previous work in this laboratory has demonstrated that SOD effectively decreased the reduction of MTT in neutrophil cultures by preventing the formation of extracellular formazan crystals by neutrophil-derived superoxide, an agent known to reduce MTT.[13] A significant decrease in MTT reduction was noted in most of the SOD-treated cultures in comparison with the comparable culture without SOD, indicating the SOD was active (Table 1). The data also demonstrate that the neutrophils used in these studies were viable and that several of the activating stimuli substantially increased metabolic activity (Table 1). Statistically significant enhancement was noted for OpZy and PMA, and LPS caused marginally significant enhancement (as determined by ANOVA and Bonferroni's test). Nitrite is a stable end-product of RNI and its concentration in 24-hr cultures was evaluated to provide an indication of cumulative RNI production. Induction of iNOS in mouse macrophages was clearly evident within 24 hr (Fig. 1), and any iNOS induction in neutrophils should have been detectable in the 24-hr time frame of the present experiments.

Nitrite production by rodent neutrophils

Populations of rodent peritoneal cells enriched for neutrophils or macrophages were obtained by harvesting the cells at different times after intraperitoneal administration of a casein solution (8 hr for neutrophil preparations and 96 hr for macrophage preparations). Because these cell preparations contained a substantial percentage of other cell types, it was not possible to determine clearly what portion of the nitrite was produced by each cell type. To explore this issue, nitrite production by mouse macrophage and neutrophil preparations was examined using different cell densities. The cells in this experiment and subsequent experiments with rats were stimulated with LPS. Unstimulated controls were included in all experiments and produced much less nitrite than LPS-stimulated cultures (see representative data for rat cell preparations on p. 139). Differential cell counts were used to assess the percentage of neutrophils and macrophages in each cell preparation, and nitrite production was plotted against the density of macrophages or neutrophils per culture (Fig. 1). When macrophage densities in the neutrophil preparation and in the macrophage preparation were plotted against nitrite concentration, the result was an essentially linear relationship. Extrapolation of a line defined by the three data-points from the macrophage preparation agreed closely with the observed plot of nitrite concentration versus macrophage density in the neutrophil preparation (Fig. 1a). This is consistent with the hypothesis that macrophages are responsible for essentially all nitrite production in both populations. This was confirmed

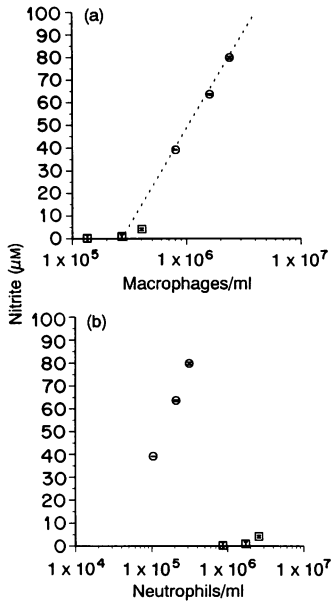

Figure 1. Assessment of the relative contributions of mouse neutrophils and macrophages to nitrite production in response to LPS (1 μg/ml). Cells were incubated for 24 hr at 37° prior to the determination of nitrite concentration in the culture medium. Both panels show the same data. In (a) nitrite production by the neutrophil preparation and the macrophage preparation are plotted versus the macrophage density in each culture; in (b) the data are plotted as nitrite concentration versus neutrophil concentration in each cell preparation. The dashed line in (a) represents the regression line determined by the data from the macrophage cell preparation. Values shown are means ± SE for triplicate culture wells. (○) Macrophage preparation (81 ± 4% macrophages, 11 ± 1% neutrophils). (□) Neutrophil preparation (87 ± 2% neutrophils, 14 ± 2% macrophages).

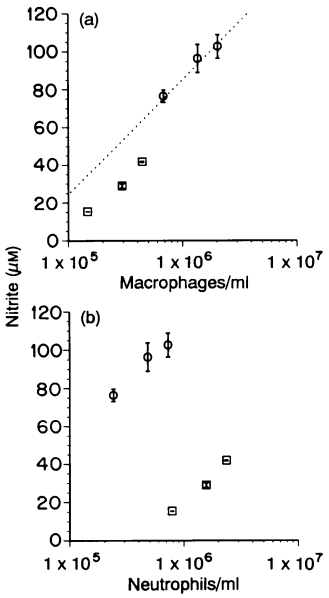

Figure 2. Assessment of the relative contributions of rat neutrophils and macrophages to nitrite production in response to LPS (1 μg/ml). Cells were incubated for 24 hr at 37° prior to the determination of nitrite concentration in the culture medium. Both panels show the same data. In (a) nitrite production by the neutrophil preparation and the macrophage preparation are plotted versus the macrophage density in each culture; in (b), the data are plotted as nitrite concentration versus neutrophil concentration in each cell preparation. The dashed line in (a) represents the regression line determined by the data from the macrophage cell preparation. Values shown are means ± SE for triplicate culture wells. (○) Macrophage preparation (68 ± 2% macrophages, 24 ± 2·6% neutrophils). (□) Neutrophil preparation (78 ± 0·6% neutrophils, 15 ± 4·9% macrophages).

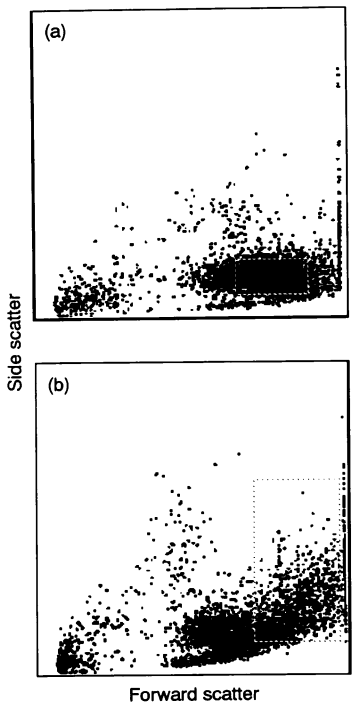

Figure 3. Enrichment of rat neutrophils and macrophages by fluorescence-activated cell sorting. The areas inside the hatched lines represent the cells that were collected during the sort of a neutrophil preparation (a) and a macrophage preparation (b). These areas were selected on the basis of the characteristic patterns and locations for mammalian leucocyte cell types. The scale for the *x*-axis is not the same for the upper and lower panels.

by plotting the same data as nitrite concentration versus neutrophil density in both cell preparations. This plot (Fig. 1b) indicated very low levels of nitrite production at high neutrophil densities, but high-level nitrite production by the macrophage preparation in which neutrophil densities were much lower. Similar results were noted in a second experiment (data not shown).

A similar experiment with rat macrophage and neutrophil preparations produced similar results (Fig. 2). Although macrophage and neutrophil preparations from rats produced higher concentrations of nitrite than preparations from mice, the patterns were similar. Extrapolation of a line defined by the three data-points from the macrophage preparation predicted slightly higher nitrite production than was actually observed in the neutrophil preparation (Fig. 2a). Thus, the macrophages in the neutrophil preparation could account for all of the nitrite observed in the neutrophil preparation, and nitrite production by these macrophages might even have been decreased slightly by the presence of neutrophils at high density. Alternatively, the actual relationship between macrophage density and nitrite concentration might best be represented by a sigmoidal curve, in which case macrophage density predicted nitrite concentration for both the macrophage and the neutrophil preparation, regardless of neutrophil density (Fig. 2a). In contrast, when nitrite concentration was plotted against neutrophil cell density, the relationship was discontinuous, with high levels of nitrite production at low neutrophil densities in the macrophage preparation and low levels of nitrite production at high neutrophil densities in the neutrophil preparation (Fig. 2b).

In an attempt to obtain more purified rat neutrophil and macrophage preparations, a fluorescence-activated cell sorter was used. Neutrophil and macrophage preparations were obtained as already described, and further purified on the basis of forward scatter (an indicator of cell size) and side scatter (an indicator of intracellular structural complexity). The cells isolated from each preparation are indicated in Fig. 3. This method provided a macrophage preparation that contained 91·25 ± 1·49% (mean ± SE for triplicate samples) macrophages and 8·75 ± 1·49% neutrophils, and a neutrophil preparation that contained 12·75 ± 1·11% macrophages and 81·75 ± 4·96% neutrophils. These preparations were only slightly purer than typical unsorted preparations, so it was not surprising that the pattern of nitrite production by sorted macrophages and neutrophils was similar to that obtained with unseparated populations (Fig. 4). Nitrite production in both preparations was substantially enhanced by LPS compared to unstimulated control cultures. Cell numbers obtained from sorting were insufficient to evaluate multiple cell densities for the macrophage preparation. However, a plot of nitrite concentration versus macrophages/ml in both the macrophage and neutrophil preparations demonstrated that macrophage

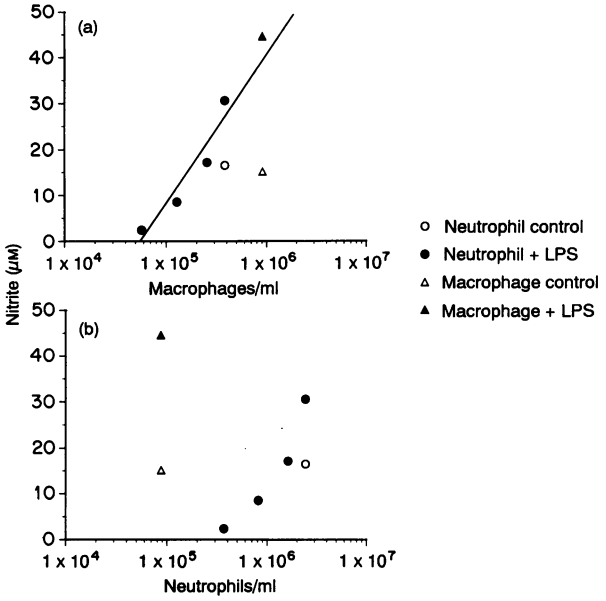

Figure 4. Assessment of the relative contributions of rat neutrophils and macrophages, enriched by fluorescence-activated cell sorting, to nitrite production by unstimulated cells (control) or in response to LPS (1 μg/ml). After sorting, cells were incubated for 24 hr at 37° prior to the determination of nitrite concentration in the culture medium. Both panels show the same data. In (a) nitrite production by the neutrophil preparation and the macrophage preparation are plotted versus the macrophage density in each culture; in (b), the data are plotted as nitrite concentration versus neutrophil concentration in each cell preparation. The solid line in (a) represents the regression line determined by the data from the neutrophil cell preparation. Values shown are means for triplicate culture wells, and standard errors were less than 10% of the mean in all cases.

density (Fig. 4a), not neutrophil density (Fig. 4b), predicted nitrite concentration.

DISCUSSION

Data presented here suggest that RNI production by mammalian neutrophils is insufficient to provide antimicrobial or anti-tumour activity. In most experiments in which RNI have been implicated as important antimicrobial or anti-tumour agents of rodent macrophages, nitrite production has exceeded 20 μM/10^6 cells/24 hr.[1–5] The rate of nitrite production by human and mouse neutrophil preparations was much less than this (Table 1 and Fig. 1). Rat neutrophil preparations produced higher concentrations of nitrite, but this was apparently due to the high rate of nitrite production by contaminating macrophages (Figs 2 and 4). Thus, it is unlikely that RNI are typically produced by mouse, rat or human neutrophils in quantities sufficient to act as effective antimicrobial or anti-tumour agents. However, it remains possible that lower quantities of RNI may be required by neutrophils than by macrophages to exert antimicrobial or anti-tumour effects. It is also possible that neutrophils produce substantial amounts of RNI but handle these compounds differently than macrophages, yielding much lower levels of nitrite.

To our knowledge only one report has noted production of nitrite by neutrophils at levels suggesting antimicrobial concentrations of RNI (~45 μM/1·5 × 10^6 cells/24 hr).[6] That study utilized male Fisher 344 rats, the same strain and sex used in the present study. Inflammatory neutrophils were isolated from surgically implanted polyvinyl alcohol sponges, and the resultant neutrophil preparations were at least 95% pure. That technique may have been more effective than the casein elicitation procedure used in the present study in the induction of RNI production by neutrophils. It is also possible that contaminating macrophages were partly responsible for the observed high levels of nitrite production. Data from the present study suggest that even 5% contamination by activated macrophages might have accounted for a portion of the reported nitrite production and that potentially antimicrobial levels of iNOS-derived RNI were not produced by rat inflammatory neutrophils elicited by casein.

The human neutrophils used in the present study were not inflammatory exudate cells such as those that were isolated from the casein-elicited mice and rats. Therefore, a variety of stimulating agents were used in an effort to induce high-level RNI production. In addition, nitrite concentrations were evaluated at 2 as well as 24 hr after stimulation, to distinguish between activation of a pre-existing enzyme (such as the Ca^{2+}-dependent nitric oxide synthase) or induction of an enzyme previously present at very low concentrations (such as iNOS). It remains possible that more complex mixtures of stimuli that might be encountered *in vivo* could induce or trigger high-level RNI production by human neutrophils. However, the data are entirely consistent with the hypothesis that mammalian neutrophils are able to produce only enough nitric oxide to act as a vasodilator or neurotransmitter, but not enough to be effective as an antimicrobial or anti-tumour agent. These data are also consistent with the results of Keller *et al.* who noted minimal nitrite production by human neutrophils during a 1-hr culture with various stimuli,[10] and with the results of Yan *et al.* who noted very low levels of conversion of arginine to citrulline by human neutrophils exposed to various cytokines and LPS.[16]

It also seemed possible that the production of little or no nitrite by human neutrophils might be caused by the formation of a different end-product, not by the production of little or no RNI. However, high-level RNI production in mouse macrophages was consistently associated with the production of nitrate and nitrite at a 1:1–2:3 ratio.[4,17] Thus, the absence of nitrite production by human neutrophils indicates either the lack of high-level RNI production or a major difference in the fate of RNI following production. This difference does not seem to be related to concomitant superoxide production by the neutrophils, because SOD did not significantly increase nitrite levels.

Data from the present study suggest that neutrophils from rats, mice and humans are similar in their inability to produce high levels of RNI. Thus, rodent neutrophils seem to be an appropriate model for human neutrophils with regard to RNI-related functions (e.g. vasodilation or microbicidal activity). However, it is important to note that this does not seem to be the case for macrophages. It is clear that rodent macrophages produce high concentrations of nitrite when induced by IFN-γ, LPS or heat-killed bacteria,[5,18] but human macrophages apparently do not.[8,9]

REFERENCES

1. KELLER R., GEIGES M. & KEIST R. (1990) L-Arginine-dependent reactive nitrogen intermediates as mediators of tumour cell killing by activated macrophages. *Cancer Res* **50,** 1421.
2. DENIS M. (1991) Interferon-gamma-treated murine macrophages inhibit growth of Tubercle Bacilli via generation of reactive nitrogen intermediates. *Cell Immunol* **132,** 150.
3. TAKEMA M., INABA K., UNO K., KAKIHARA K.-I., TAWARA K. & MURAMATSU S. (1991) Effect of L-arginine on the retention of macrophage tumouricidal activity. *J Immunol* **146,** 1928.
4. ADAMS L.B., HIBBS J.B., TAINTOR R.R. & KRAHENBUHL J.L. (1990) Microbistatic effect of murine-activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-arginine. *J Immunol* **144,** 2725.
5. KELLER R., GEHRI R., KEIST R., HUF E. & KAYSER F.H. (1991) The interaction of macrophages and bacteria: a comparative study of the induction of tumouricidal activity and of reactive nitrogen intermediates. *Cell Immunol* **134,** 249.
6. ALBINA J.E., MILLS C.D., HENRY W.L. & CALDWELL M.D. (1990) Temporal expression of different pathways of L-arginine metabolism in healing wounds. *J Immunol* **144,** 3877.
7. NUSSLER A.K. & BILLIAR T.R. (1993) Inflammation, immunoregulation, and inducible nitric oxide synthase. *J Leuk Biol* **54,** 171.
8. PADGETT E.L. & PRUETT S.B. (1992) Evaluation of nitrite production by human monocyte-derived macrophages. *Biochem Biophys Res Commun* **186,** 775.
9. CAMERON M.L., GRANGER D.L., WEINBERG J.B., KOZUMBO W.J. & KOREN H.S. (1990) Human alveolar and peritoneal macrophages mediate fungistasis independently of L-arginine oxidation to nitrite or nitrate. *Am Rev Respir Dis* **14,** 1313.
10. KELLER R., KEIST R., ERB P. *et al.* (1990) Expression of cellular effector functions and production of reactive nitrogen intermediates: a comparative study including T lymphocytes, T-like cells, neutrophil granulocytes, and mononuclear phagocytes. *Cell Immunol* **131,** 398.
11. SCHMIDT H.H.H.W., SEIFERT R. & BÖHME E. (1989) Formation and release of nitric oxide from human neutrophils and HL-60 cells induced by a chemotactic peptide, platelet activating factor and leukotriene B4. *FEBS Lett* **2,** 357.
12. MALAWISTA S.E., MONTGOMERY R.R. & VAN BLARICOM G. (1992) Evidence for reactive nitrogen intermediates in killing of staphylococci by human neutrophil cytoplasts. A new microbicidal pathway for polymorphonuclear leukocytes. *J Clin Invest* **90,** 631.
13. PRUETT S.B. & LOFTIS A.Y. (1990) Characteristics of MTT as an indicator of viability and respiratory burst activity of human neutrophils. *Int Arch Allergy Appl Immunol* **92,** 189.
14. MCCALL T.B., BOUGHTON-SMITH N.K., PALMER R.M.J., WHITTLE B.J.R. & MONCADA S. (1989) Synthesis of nitric oxide from L-arginine by neutrophils. *Biochem J* **261,** 293.
15. MOSMANN T. (1983) Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assays. *J Immunol Meth* **65,** 55.
16. YAN L., VANDIVIER R.W., SUFFREDINI A.F. & DANNER R.L. (1994) Human polymorhonuclear leukocytes lack detectable nitric oxide synthase activity. *J Immunol* **153,** 1825.
17. DRAPIER J.-C. & HIBBS J.B. (1988) Differentiation of murine macrophages to express nonspecific cytotoxicity for tumour cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. *J Immunol* **140,** 2829.
18. HIGGINBOTHAM J.L., LIN T.L. & PRUETT S.B. (1992) Effect of macrophage activation on killing of *Listeria monocytogenes*: roles of reactive oxygen or nitrogen intermediates, rate of phagocytosis, and retention of bacteria in endosomes. *Clin Exp Immunol* **88,** 492.