

some inherited disorders such as juvenile Huntington's chorea and congenital myotonic dystrophy.<sup>23</sup> Wilkins<sup>25</sup> suggested a modification of Knudson's two-mutational-event theories involving genomic imprinting which would explain the non-random retention of the paternal allele of chromosome 11 in Wilms tumour.

Genomic imprinting satisfactorily accounts for the transmission of glomus tumours in the pedigrees in our study and those previously reported. We suggest that the autosomal dominant gene, which is inactivated during female oogenesis, with the result that the first generation is not affected, can be reactivated after demethylation during spermatogenesis and lead to affected offspring in the second generation. This hypothesis would explain the pedigrees where unaffected males, with affected mothers or maternal grandmothers, had several affected children. There were a few instances of transmission of the gene through apparently unaffected males in two generations (fig 3) which might have occurred because the gene escaped activation during male spermatogenesis or the tumour was present but undetected. Only once a "carrier" female was said to be unaffected (fig 4).

Genomic imprinting also offers an explanation for the female preponderance in non-familial patients, which is very pronounced for such a heterogeneous group. Very early post-zygotic mutations can produce germline mosaicism.<sup>26</sup> Some females are thought to have sporadic glomus tumour because the heritable character is not apparent until the mutation, passed through a paternal germline, is expressed in the second or later generation (fig 1). In contrast, males are more often identified as having an heritable tumour as the disease becomes manifest in the next generation (figs 1 and 2). In addition some patients represent true sporadic post-zygotic mutations.

Genomic imprinting is consistent with the extensive pedigree data which we present. Similar explanations have been proposed for the non-random retention of alleles in retinoblastoma and Wilms tumour.<sup>25,27,28</sup> Genomic imprinting may be a more general phenomenon in genetic tumour susceptibility than previously recognised. The involvement of genomic imprinting, derived from allele retention in carcinogenesis is still a matter of much discussion<sup>29</sup>—our pedigree studies provide strong and independent evidence for a role in the control of cell growth.

These findings are highly relevant for genetic counselling. The risk for the offspring of affected males remains 50%, for those of affected females the risk is very low, and for the children of sons of women with glomus tumours the risk is 25%. The grandchildren or great-grandchildren of female patients who are thought to have sporadic tumours may also be at risk. DNA studies are needed to confirm the genomic imprinting hypothesis in relation to glomus tumours and other hereditary diseases.

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## SYSTEMIC GLUTATHIONE DEFICIENCY IN SYMPTOM-FREE HIV-SEROPOSITIVE INDIVIDUALS

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**Summary** To find out whether systemic glutathione deficiency is associated with human immunodeficiency virus (HIV) infection, thus contributing to the immunodeficiency state, glutathione concentrations in venous plasma and lung epithelial lining fluid (ELF) of symptom-free HIV-seropositive and normal individuals were measured. Total and reduced glutathione concentrations in the plasma of the HIV-infected subjects were about 30% of those in the normal individuals. Concentrations of these substances in the ELF of HIV-infected subjects were about 60% of those in the controls. There was no correlation between ELF and plasma concentrations of total or reduced glutathione. Since glutathione enhances immune function, glutathione deficiency may contribute to the progressive immune dysfunction of HIV infection.

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## Introduction

DEPLETION of CD4+ T-cells plays an important part in the pathogenesis of the acquired immunodeficiency syndrome.<sup>1-3</sup> However, various abnormalities of the functions of T-cell subsets, B-cells, and cells of monocyte/macrophage lineage observed in symptom-free individuals seropositive for the human immunodeficiency virus (HIV) and having normal numbers of CD4-positive T-cells, suggest that other mechanisms contribute to the development of the early immune dysfunction associated with HIV infection.<sup>4,5</sup> Further, although it is now clear that symptom-free HIV-seropositive individuals have evidence of the HIV-virus in lymphocytes and macrophages,<sup>6,7</sup> the number of infected cells cannot account for the range of immune abnormalities observed in these individuals.

Glutathione (L- $\gamma$ -glutamyl-L-cysteinyl-glycine, GSH) may contribute to this process.<sup>8-10</sup> Extracellular glutathione protects cells of diverse tissues against oxidation injury and is the major transport form of the amino acid cysteine. Within cells, glutathione is the dominant intracellular thiol, where it functions directly or indirectly as an antioxidant, in reactions involving the synthesis of proteins and DNA precursors, and as a cofactor for various enzymes. Glutathione is also believed to be important in the initiation and progression of lymphocyte activation, and thus essential for host defence.<sup>11-15</sup> Furthermore, depletion of intracellular glutathione inhibits lymphocyte activation by mitogens, and glutathione is critical for the function of natural killer cells and for lymphocyte-mediated cytotoxicity.<sup>11-14</sup>

To assess whether the symptom-free HIV-seropositive state may be associated with a global deficiency of extracellular glutathione concentrations of this substance were measured in plasma and lung epithelial lining fluid (ELF), an easily obtainable fluid from an organ that is commonly affected in AIDS.

## Subjects and Methods

### Subjects

The subjects were 14 symptom-free HIV-seropositive men (mean age 35 [SEM 2] years). HIV infection was confirmed by enzyme-linked immunosorbent assay (ELISA) and western blot analysis. At the time of study none fulfilled Centers for Disease Control criteria for AIDS-related complex or AIDS (ie, all were group II).<sup>16</sup> All were homosexual and 3 of them were also intravenous drug abusers. None were smokers. 10 were untreated, and 4 were receiving zidovudine. Mean lymphocyte count was  $804 \times 10^3$  cells/ $\mu$ l ( $346 \times 10^3$  CD4+ lymphocytes/ $\mu$ l). p24 antigen was not detected in the sera of any individual. 5 of the 14 subjects had serological evidence of exposure to hepatitis B virus; of these, 1 had mildly abnormal liver function. All had normal chest X-rays. In average, lung function tests were normal (see accompanying table), but 4 of 14 individuals had a mild reduction in one of the indices of lung function.

The controls were 19 healthy HIV-seronegative (by ELISA) individuals (14 male, 5 female, mean age 36 [SEM 3] years). All were non-smokers and all were free of lung disease as determined by history, physical examination, chest X-ray, and pulmonary function tests (table).

Both subjects and controls gave informed consent for the study, including bronchoalveolar lavage. The study protocol (77-H-61) was approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute.

### Biological Samples

Venous plasma and bronchoalveolar lavage (BAL) fluid were obtained by standard techniques. Portions of the BAL fluid were

## LUNG FUNCTION AND BAL FLUID FINDINGS

	Normal	HIV-seropositive
<i>Lung function* (% predicted)</i>		
Vital capacity	92 (3)	90 (4)
FEV <sub>1</sub>	101 (4)	96 (7)
FEV <sub>1</sub> /FVC	110 (3)	108 (3)
DLCO	104 (5)	88 (4)
<i>Bronchoalveolar lavage</i>		
% fluid recovered	64 (3)	62 (4)
Total cells recovered ( $\times 10^3$ /ml BAL fluid)	137 (22)	265 (52)
Cell differential (%)		
Macrophages	89 (2)	71 (4)
Lymphocytes	9 (2)	28 (3)
Neutrophils	1 (1)	1 (0.3)
Eosinophils	<1	<1
CD4+ /CD8+ T-cells†	ND*	0.3 (0.1)

\*Numbers in parentheses refer to SEM.

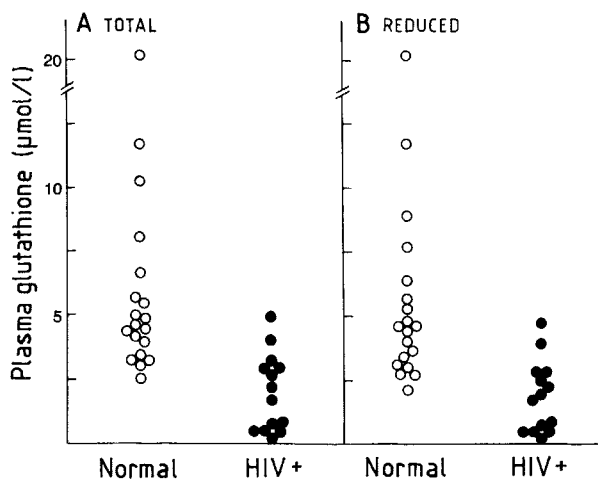
FEV<sub>1</sub>=forced expiratory volume in 1 s; FVC=forced vital capacity; DLCO = diffusion capacity for carbon monoxide, corrected for haemoglobin and volume; see reference 37 for lung function methods and predicted values; ND = not done.

†Ratio of CD4+ to CD8+ T-cells quantified by flow cytometry.<sup>38</sup>

taken for glutathione assays (see below), for cell counts (total cell and T-lymphocyte CD4+ and CD8+ subtypes), and for cytocentrifuge preparations for differential counts. The cells were pelleted and the supernatant was used to quantify the amount of ELF recovered (see below). For a variety of reasons unrelated to this study, not all subjects and controls could undergo lavage.

### Glutathione Concentrations and Form

Glutathione concentrations in venous plasma and bronchoalveolar lavage fluid were quantified with minor modifications of standard methods.<sup>17-19</sup> All measurements were made in duplicate and the average value calculated. In brief, to determine the total glutathione concentrations (reduced glutathione plus glutathione disulphide) venous blood or lavage fluid was mixed with an equal amount of 10 mmol/l 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) in 0.1 mol/l potassium phosphate, pH 7.5, containing 17.5 mmol/l edetic acid. The samples were centrifuged (2000 g, 10 min), and portions (50  $\mu$ l) of the supernatants were added to cuvettes containing 0.5 U of glutathione disulphide (GSSG) reductase in 0.1 mol/l potassium phosphate, pH 7.5, containing 5 mmol/l edetic acid. After incubation for 1 min at 25°C, the assay reaction was started by adding 220 nmol/l of the reduced form of  $\beta$ -nicotinamide adenine dinucleotide phosphate in 0.1 mol/l potassium phosphate, pH 7.5, containing 5 mmol/l edetic acid in a final volume of 1 ml. The rate of reduction of DTNB was recorded spectrophotometrically at a wavelength of 412 nm with a Beckman DU-70 spectrophotometer. Determination of the total glutathione concentration was based on standard curves generated from known concentrations of GSSG (0.125 to 4  $\mu$ mol/l) in phosphate buffered saline, pH 7.4. To quantify glutathione disulphide, venous blood or lavage fluid was mixed, immediately after recovery, with an equal volume of 10 mmol/l N-ethylmaleimide (NEM) in 0.1 mol/l potassium phosphate, pH 6.5, containing 17.5 mmol/l edetic acid. The samples were then centrifuged at 2000 g for 10 min, and 250  $\mu$ l of the supernatant was passed through a 'SEP-PAK C18' cartridge (Waters Associates) that had been washed with 3 ml methanol followed by 3 ml water, and the effluent was collected. GSSG was eluted from the column with 1 ml of 0.1 mol/l potassium phosphate, pH 7.5, containing 5 mmol/l edetic acid. A portion (750  $\mu$ l) of the combined effluent and eluate was mixed with 250  $\mu$ l 0.1 mol/l potassium phosphate, pH 7.5, containing 5 mmol/l edetic acid, 800  $\mu$ mol/l DTNB, 2 U/ml glutathione reductase, and 1 mmol/l NADPH, and the rate of reduction of DTNB was recorded spectrophotometrically at 412 nm. Standard curves were derived from dilutions of known concentrations of GSSG (0.125 to 4  $\mu$ mol/l) that had been mixed with 10 mmol/l NEM and chromatographed with SEP-PAK C18 cartridges as described above. The amount of glutathione was obtained by subtracting the



**Fig 1—Plasma glutathione concentrations in normal controls and symptom-free HIV-seropositive individuals.**

A—total glutathione (GSH + 2GSSG) concentrations; B—reduced glutathione (GSH) concentrations. Each point represents the average of duplicate measurements for each individual.

amount of GSSG from the total glutathione levels. The ELF total and reduced glutathione levels were calculated with reference to the volume of ELF recovered as assessed by the urea method.<sup>20</sup>

#### Statistical Analysis

All data are presented as mean (SEM); all statistical comparisons were made with the two-tailed Student's *t*-test.

### Results

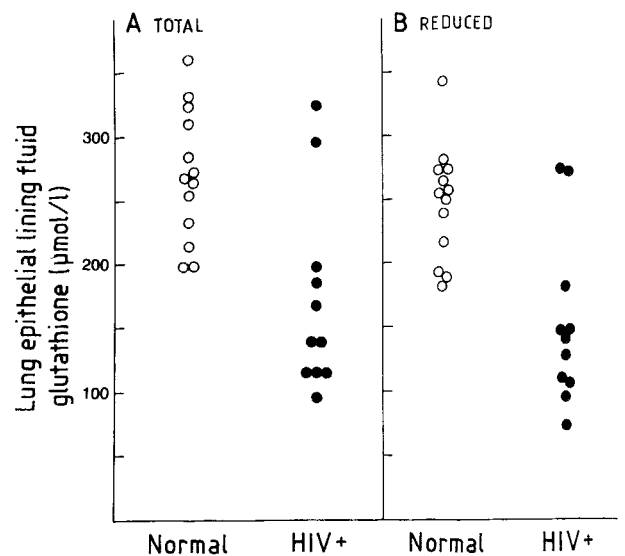
#### Plasma Glutathione

Mean plasma total glutathione concentration in normal controls was 5.99 (0.97) µmol/l (fig 1A). Of this 5.71 (0.97) µmol/l was in the form of reduced glutathione—ie, 94.0 (1.3)% was in the functional form (fig 1B). Plasma total and reduced glutathione concentrations in the HIV-seropositive group were about a third of those in the controls—mean total glutathione 1.97 (0.41) µmol/l and reduced glutathione 1.83 (0.38) µmol/l ( $p < 0.005$  for both total and reduced). As in the controls the plasma glutathione in the HIV-seropositive group was almost entirely (95.3 [1.6]%) in the reduced form.

#### Bronchoalveolar Lavage

Although the HIV-seropositive individuals had normal numbers of blood lymphocytes, the proportion of lymphocytes in their lavage fluid (28 [3]%) was significantly higher than that in controls (9 [2]%,  $p < 0.001$ ), as was the total number of cells recovered by lavage (HIV-seropositive  $265 [52] \times 10^3$  cells/ml lavage fluid, controls  $137 [22] \times 10^3$  cells/ml,  $p < 0.05$ )—ie, the HIV-seropositive individuals had a lymphocytic alveolitis although they had no symptoms referable to the lung and their chest X-rays and lung function were normal or close to normal. Their ratio of CD4+ to CD8+ T-cells in the lavage fluid was very low (0.3 [0.1]).

As in plasma, concentrations of glutathione in ELF were lower in HIV-seropositive individuals than in controls. As expected,<sup>19</sup> in normal controls total glutathione in ELF (270 [14] µmol/l, fig 2A) was approximately 45 times higher than that in plasma. The ELF glutathione was almost all in the reduced form (245 [12] µmol/l, or 91.1 [1.5]% of total glutathione, fig 2B). ELF total glutathione (170 [23] µmol/l) and reduced glutathione (151 [20] µmol/l or 88.6 [2.5]% of



**Fig 2—Lung epithelial lining fluid glutathione concentrations in normal individuals and symptom-free HIV-seropositive subjects.**

A—total glutathione (GSH + 2GSSG) levels; B—reduced glutathione (GSH) concentrations. Each point represents the average of two measurements for each individual.

total glutathione) in HIV-seropositive subjects were about 60% of those in normal individuals. For two of the HIV-seropositive individuals, the total and reduced glutathione levels in ELF overlapped with those of the controls (fig 2). There were no clinical or laboratory features that distinguished these two individuals from the others.

There was no relation between ELF and plasma total or reduced glutathione concentrations, so the relative glutathione deficiency in HIV-seropositive individuals is a generalised process occurring in both blood and lung compartments.

### Discussion

Several lines of evidence support the concept that systemic glutathione deficiency could be one of several factors responsible for immune deficiency in HIV infection. First, glutathione is the major form of transport in plasma for cysteine, the major plasma sulphhydryl compound, and a major antioxidant in plasma,<sup>8-10,21</sup> and sulphhydryl compounds augment the activation of cytotoxic T cells in mixed lymphocyte cultures, T-cell proliferation in response to mitogens, and the differentiation of T and B lymphocytes.<sup>11-14,22</sup> Furthermore, in-vivo administration of glutathione enhances activation of cytotoxic T-cells.<sup>15</sup> Also, natural killer activity, lymphocyte proliferation in response to mitogens, and generation of immunoglobulin secreting cells are all susceptible to injury by extracellular oxidants, and exposure of lymphocytes to extracellular oxidants causes breaks in DNA strands and suppression of the ability of lymphocytes to proliferate.<sup>23,24</sup>

Second, observations that depletion of intracellular glutathione inhibits the activation of lymphocytes, increases susceptibilities of human lymphoid cells to radiation, and suppresses cell-mediated cytotoxic functions<sup>11-14,25</sup> suggest that intracellular glutathione can modulate the function of immune cells.

Third, investigators routinely add the sulphhydryl reducing agent 2-mercaptoethanol to mouse lymphocyte cultures to enhance survival and to augment proliferation and function,<sup>26</sup> and RPMI 1640, a medium routinely used in human lymphocyte cultures, contains 3 µmol/l glutathione.

Fourth, the relative glutathione deficiency in the ELF may have particular relevance to the fact that the lung is the major site of opportunistic infections in AIDS. Whereas blood lymphocyte numbers in symptom-free HIV-seropositive individuals are normal or reduced,<sup>1-3</sup> the lung lymphocyte numbers in the BAL fluid of our study population, and in other reports of HIV-seropositive individuals,<sup>27-29</sup> were very high. Since lymphocytes require extracellular cysteine to maintain intracellular glutathione levels, and since extracellular glutathione is the main source of extracellular cysteine, the glutathione deficiency in ELF of HIV-seropositive individuals may be potentiated by the increased numbers of lymphocytes.

Finally, the observation that the HIV-seropositive state is associated with glutathione deficiency in plasma and in the lung makes it, to our knowledge, the only known condition in which there is a generalised deficiency in extracellular glutathione levels. Idiopathic pulmonary fibrosis (IPF), as a chronic inflammatory lung disorder, is associated with a local (lung) deficiency of glutathione, but the plasma levels of glutathione in IPF are normal.<sup>30</sup> Furthermore, the glutathione abnormalities observed in the lung in HIV-seropositive individuals are unlikely to be associated with non-specific inflammation since cigarette smokers have increased lung glutathione levels (and normal plasma levels) despite the presence of activated alveolar macrophages and the accumulation of neutrophils in the lower respiratory tract.<sup>19,31</sup>

What produces the systemic glutathione deficiency in symptom-free HIV-seropositive individuals is unknown. The deficiency could be due to any combination of decreased synthesis, increased breakdown, and increased utilisation. Glutathione is synthesised within most cells, but not all cells secrete it.<sup>8-10,21</sup> It is believed that most plasma glutathione is provided by the liver,<sup>8-10,32</sup> and current evidence suggests that the bulk of plasma glutathione is metabolised by the kidney and possibly the lung.<sup>8-10,21,33</sup>

Although AIDS patients do have liver abnormalities, liver function must be grossly abnormal to cause plasma glutathione concentrations to drop.<sup>33,34</sup> 5 of our 14 HIV-seropositive subjects had evidence of exposure to the hepatitis B virus, but only 1 had even mild liver disease. It is conceivable that  $\gamma$ -glutamyltransferase levels in organs such as the kidney and lung might be raised, but the global nature of the glutathione deficiency would necessitate postulating mediator(s) from infected cells that would modulate this enzyme system.

Whatever the mechanism producing the systemic glutathione deficiency, our findings are consistent with the report that AIDS patients have low plasma cysteine concentrations and low acid-soluble thiol concentrations,<sup>35</sup> since glutathione is the major source of plasma cysteine and low molecular weight thiols.<sup>8-10</sup> Further, since lymphocytes require extracellular cysteine to maintain intracellular glutathione concentrations, and since intracellular glutathione is necessary for lymphocyte activation,<sup>11-15</sup> it is relevant that AIDS patients have reduced intracellular concentrations of glutathione in blood mononuclear cells<sup>35</sup>—ie, the systemic extracellular glutathione deficiency may dictate a relative intracellular glutathione deficiency in cells of the immune system.

Several considerations are relevant to the question of whether glutathione or molecules with analogous function may be useful in the therapy of HIV infection. First, it is not yet clear whether the abnormalities in immune function

relate to glutathione in the metabolism of immune cells, or its function as an antioxidant. If glutathione influences metabolism, treatment with glutathione or a close analogue might be useful, whereas if it is the antioxidant function that is relevant, a variety of molecules might be useful.

Second, it is not clear whether the systemic extracellular glutathione deficiency directly affects immune function, or whether it is a manifestation of diminished intracellular glutathione concentrations—so should therapy be directed extracellularly, intracellularly, or both? Finally, if the extracellular systemic deficiency of glutathione observed in the present study does contribute to the global immunodeficiency associated with HIV infection, a central problem is how to re-establish normal extracellular concentrations of glutathione. GSH given intravenously has a half life in plasma of only 1.6 minutes.<sup>36</sup> For organs such as the lung, direct delivery by aerosol inhalation might be suitable.<sup>37</sup> Alternatively, analogues of GSH such as glutathione esters might be used, as has been done in animals, with a marked increase in lymphocyte GSH levels.<sup>33</sup>

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## ENDOTHELIAL CELL ACTIVATION AND HIGH INTERLEUKIN-1 SECRETION IN THE PATHOGENESIS OF ACUTE KAWASAKI DISEASE

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**Summary** To investigate the pathogenesis of Kawasaki disease, the effects of intravenous gammaglobulin treatment on circulating cytotoxic antibodies against endothelial cells, in-situ endothelial cell activation, and cytokine production and action were examined. Gammaglobulin treatment did not reduce cytotoxic antibody activity against endothelial cells in six patients tested. Expression of endothelial cell activation antigens (endothelial-leucocyte adhesion molecule-1 [ELAM-1] and intercellular adhesion molecule-1) was detected by means of immunoperoxidase staining in skin biopsy samples from five patients before treatment. Samples were obtained immediately after treatment from six patients; there was no endothelial cell activation in four and the two with persistent activation had persistent fever and mucocutaneous symptoms. Peripheral blood mononuclear cells from ten of sixteen acute Kawasaki disease patients spontaneously secreted high levels of interleukin-1 (IL-1). IL-1 secretion remained high in four gammaglobulin-treated patients in whom coronary artery abnormalities developed but fell to normal in six treated patients who had no coronary artery abnormalities. In cell culture, gamma globulin did not inhibit endothelial cell expression of ELAM-1 in response to IL-1 or tumour necrosis factor. The association between improvement of clinical symptoms and the reduction of cytokine secretion and reversal of endothelial cell activation supports a role for immune-mediated injury to cytokine-induced endothelial cell antigens in the pathogenesis of this disorder.

## Introduction

ALTHOUGH Kawasaki disease, an acute vasculitis of unknown aetiology that primarily affects young children,<sup>1,2</sup> is self-limited, serious sequelae can occur. Coronary artery aneurysms or ectasia develop in 15–25% of patients and may lead to myocardial infarction, sudden death, or chronic coronary artery insufficiency.<sup>3–5</sup> In the United States and Japan, Kawasaki disease is a leading cause of acquired heart disease in children.

Several factors are likely to be involved in the pathogenesis of Kawasaki disease. However, several observations suggest a role for immune system abnormalities. The disease is associated with a diffuse vasculitis of small and medium blood vessels.<sup>6,7</sup> The acute phase is characterised by an immunoregulatory imbalance that consists of a deficiency of suppressor/cytotoxic T cells, high numbers of activated helper T cells and monocytes, and polyclonal B-cell activation.<sup>8,9</sup> This immune activation is accompanied by high serum levels of interleukin-1 (IL-1),<sup>10</sup> tumour necrosis factor (TNF),<sup>11</sup> and interferon-gamma (IFN- $\gamma$ ),<sup>12</sup> as well as the appearance of cytotoxic antibodies against cytokine-stimulated, but not untreated, vascular endothelial cells.<sup>13,14</sup> These antibodies are not detectable in the late convalescent phase.

Cytokines can induce or increase expression of specific cell surface antigens on cultured human vascular endothelial cells.<sup>15–20</sup> These antigenic changes correlate with, and may underlie, specific cytokine-induced functional changes, such as greater leucocyte adhesion and antigen presentation. Increased expression of surface antigens has also been detected immunohistochemically on human endothelial cells in vivo, as a measure of endothelial activation.<sup>21</sup>

High-dose intravenous gammaglobulin treatment is effective in reducing the prevalence of coronary artery aneurysms in Kawasaki disease.<sup>22–24</sup> Treatment with intravenous gammaglobulin plus aspirin reduced the T-cell and B-cell activation associated with the disease.<sup>25</sup> In contrast, treatment with aspirin alone was associated with persistent T-cell and B-cell activation.

We postulated that there are two requirements for endothelial cell injury in Kawasaki disease. First, that raised cytokine production, triggered by an unknown aetiological agent, induces new endothelial cell antigens. Secondly,

## R. BUHL AND OTHERS: REFERENCES—continued

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