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Are the cellular immune responses of children and adults with *Schistosoma mansoni* infection intrinsically different? Cytokines produced *ex vivo* in response to antigens and mitogens

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SUMMARY

In recently exposed communities, intensity of schistosomiasis infection increases as children age and then drops again in adulthood, indicating that host maturity is an important aspect of resistance to schistosomiasis. We investigated whether the cellular immune response to the parasite was correlated with age in subjects with similar daily patterns of exposure, current intensities of infection and number of years of exposure.

The cellular immune response of subjects with either 'low' (under 200 eggs per gram (EPG)) or 'high' (over 400 EPG) intensities of infection was investigated, in a recently established focus where subjects had similar histories of exposure and number of years of experience with Schistosoma mansoni*. Subject's whole blood was cultured with adult worm antigen (AWA), a mixture of phytohaemagglutinin (PHA) and lipopolysaccharide (LPS), or left unstimulated, and culture supernatants were tested for IL-4, IL-5, IL-10 and IFN-γ.*

Children and adults tended to respond differently to schistosome antigen. The most statistically significant illustration of this was the negative correlation between age and IL-5 produced by samples from people with low intensities of infection cultured with AWA ($P < 0{\cdot}003$, $P < 0{\cdot}05$ *after Bonferroni correction). IL-10 produced by samples cultured with PHA and LPS was also notably lower in children than in adults, although not formally significant after Bonferoni correction.*

This indicates that it is possible for age, independently of intensity of infection or experience with the parasite, to influence the immune response to schistosomiasis.

Keywords *human, IFNγ, IL-10, IL-4, IL-5,* Schistosoma mansoni*, Senegal*

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INTRODUCTION

In endemic areas intensity of schistosomiasis infection increases as children age, to a peak between 8 and 15 years and then drops in adulthood (1). This pattern holds even in recently exposed communities where children and adults have been exposed for the same number of years and have had similar levels of day to day exposure (2), indicating that host maturity is an important aspect of resistance to schistosomiasis independent of resistance acquired through cumulative experience of infection (3,4). The maturation of the immune system could therefore play an important role in the development of resistance to schistosomiasis, as has been indicated for malaria (5) and *Helicobacter pylori* (6). This report presents a description of the cellular immune response of children and adults, in a recently established focus given similar histories of exposure and current intensities of infection.

MATERIALS AND METHODS

Subjects

Subjects were recruited from Rone and Ravette, villages situated to the north-east of Saint Louis, Senegal in an *S. mansoni* focus that could not have been more than 3 years old (2,4). Intensity of infection with *S. mansoni* was determined using egg counts in 2×25 mg kato slides from each of two stools taken on different days (7) and people were then treated. 'Children' are defined as between 8 and 15 years, and 'adults' were over 20 years. 'Low' intensity of infection was defined as lower than 200 eggs per gram of faeces (EPG), and 'high' as over 400 EPG. These cut-off levels were chosen such that there were no significant differences in intensity of infection between adults and children within the same intensity of infection group (tested using Mann–Whitney

U-tests). Low intensity of infection children ($n = 8$) had a median age of 12 years (inter-quartile range (IQR): 10–13), and a median intensity of infection of 120 EPG (IQR: 80–160), low intensity adults ($n = 30$) had a median age of 31 years (IQR: 24–54) and a median intensity of infection 20 EPG (IQR: 0–70); the median age of high intensity children ($n = 30$) was 9 years (IQR: 11–12) and the median intensity of infection 1690 EPG (IQR: 840–2270); the median age of high intensity adults ($n = 8$) was 50 years (IQR: 35–57) and the median intensity of infection 1375 EPG (IQR: 820–2140).

Culture techniques

Whole blood was cultured with 15 μg/mL of adult worm antigen (AWA), a mixture of 10 μg/mL phytohaemagglutinin (PHA) and 25 μg/mL lipopolysaccharide (LPS), or left unstimulated as a measure of spontaneous cytokine production. The combination and concentrations of PHA and LPS were as described previously (8,9). Blood was diluted 1 : 10 in culture medium and incubated in a humidified environment at 37°C and 5% CO_2. Culture medium was sterile Iscove's Modified Dulbecco's medium (Merck cat. no. 42200-014) supplemented with 100 μ/mL penicillin, 100 μg/mL streptomycin, and 3·024 g/L sodium bicarbonate, pH balanced to 7·2. Supernatants were harvested at 48, 72 and 96 h post-incubation to allow for differing times of production between the different cytokines. Supernatants were frozen at –30°C and transported to Antwerp on dry ice.

Cytokine detection

Supernatants were tested for IL-4, IL-5, IL-10 and IFNγ with sandwich ELISAs using Pharmingen antibody pairs as previously described (10). A standard curve was run on each plate using recombinant human cytokine standards as recommended by NIBSC: IL-4: NIBSC 88/656, 1000 international units/ampoule (≈ 100 ng), IL-5: NIBSC 90/586, 5000 units/ampoule (≈ 500 ng), IL-10: NIBSC reference preparation 5000 units/ampoule (≈ 1 μg/mL) and IFNγ: NIBSC 88/606, 3300 international units/ampoule (≈ 175 ng/mL). For AWA stimulated samples, the ELISA protocol was modified to measure low levels of IL-10 and IL-4, by changing the substrate from ABTS(2,2′azino-bis(3-ethyl benzthiazoline-sulphonic acid) to TMB(3,3′5,5′-tetramethylbenzidine). The sensitivity of the assays increased from 0·06 IU/mL (IL-4) and 0·08 (IL-10) to 0·02 IU/mL (IL-4 and IL-10). The sensitivity of the IL-5 assay was 0·6 IU/mL and IFNγ: 1·7 IU/mL. Results are given for the peaks of cytokine production. For cultures stimulated with AWA, peaks of production were 48 h for IL-4 and IL-5 and 96 h for IL-10 and IFNγ. For cultures stimulated with PHA/LPS, peaks of production were 48 h for IL-4 and IL-10 and 96 h for IL-5 and IFNγ.

Statistical analysis

Although this report is principally descriptive, some simple statistics have been used. In common with some other studies (11–13) some samples, particularly non-stimulated or antigen-stimulated samples, contained no detectable cytokine. This could reflect both the low frequency of antigen-specific cells in the intravascular compartment and insensitivity of the assays available to measure the cytokines. For all cytokines, the percentage of samples from each group containing detectable cytokine was compared using a Fisher's exact test. Eight such comparisons were made. Significant differences in this kind of analysis were found for IL-5 and IL-10 produced by samples cultured with AWA. Age was tested for correlation with cytokine levels, including samples containing no detectable cytokine, using Spearman's rank correlations. Sixteen correlations were carried out in total. The *P*-values quoted first are for a single correlation or Fisher's exact, the second *P*-value has been corrected for multiple testing using a sequential Bonferroni technique (14). Differences in levels of cytokine produced according to sex were also investigated, but none were found (data not shown).

RESULTS

IL-4 AWA

In the high intensity of infection category, 39% of blood samples from children produced IL-4 compared to 83% of blood samples from adults. In blood from people with a low intensity of infection, 40% of samples from children and 33% of samples from adults produced detectable cytokine. These differences were not significant. The median levels overall, and the median levels calculated only from samples producing detectable IL-4, were higher in adults compared to children in both low and high intensity of infection categories (Table 1a,b). Concentration of IL-4 correlated positively with age ($P < 0·004$) if people had intensities of infection over 400 EPG, but not if they had under 200 EPG. This result was no longer significant after Bonferroni correction, however ($P = 0·06$).

IL-4 PHA and LPS

78% of blood samples from children produced IL-4 compared to 100% of blood samples from adults in the high intensity of infection category. In the low intensity of infection category, 100% of samples from children and 87% of samples from adults produced detectable cytokine. These differences

were not significant. As for samples cultured with AWA, median levels overall and the median levels calculated only from samples producing detectable IL-4 were higher in adults compared to children in both intensity of infection categories (Table 1a,b). Again IL-4 concentration correlated positively with age ($P < 0{\cdot}02$) if people had intensities of infection over 400 EPG, but not if they had under 200 EPG, but again this result was not significant after Bonferroni correction ($P = 0{\cdot}2$).

IL-5 AWA

The percentages of samples producing IL-5 differed significantly between the four groups ($P < 0{\cdot}001$: $P < 0{\cdot}008$ after Bonferroni correction). A very low percentage of blood samples from adults (3%) with low intensities of infection produced cytokine compared with the other three groups (80% in children with a low intensity of infection, 35% in children with a high intensity of infection and 67% in adults

Table 1 The number of samples tested (*N*), percentage of samples in which cytokine was detected, the median level of cytokine in those samples in which cytokine could be detected and the median, including samples in which cytokine levels were below the detection limit of the assay for IL-4 (a, b), IL-5 (c, d), IL-10 (e, f) and IFNγ (g, h). Results are reported for children (8–15) and adults (over 20 years) with either low intensities of infection (under 200 EPG) or high intensities of infection (over 400 EPG); sample sizes are therefore slightly smaller than those quoted in Figure 1

(a) IL-4 – low intensity of infection

	No stimulant		AWA		No stimulant		PHA & LPS	
	Child	Adult	Child	Adult	Child	Adult	Child	Adult
N	5	30	5	30	5	30	5	30
Percentage with detectable cytokine	20	27	40	33	0	13	100	87
Median of those with detectable cytokine	0·6	0·05	0·1	0·07		0·4	0·3	0·5
Median overall	–	–	–	–	–	_	0·3	0·4

(b) IL-4 – high intensity of infection

	No stimulant		AWA		No stimulant		PHA & LPS	
	Child	Adult	Child	Adult	Child	Adult	Child	Adult
N	23	6	23	6	23	6	23	6
Percentage with detectable cytokine	4	17	39	83	15	17	78	100
Median of those with detectable cytokine	0·05	0·1	0·08	0·1	0·3	0·09	0·3	0·6
Median overall	–	–	–	0·1	–	–	0·3	0·6

(c) IL-5 – low intensity of infection

	No stimulant		AWA		No stimulant		PHA & LPS	
	Child	Adult	Child	Adult	Child	Adult	Child	Adult
N	5	30	5	30	8	23	8	23
Percentage with detectable cytokine	0	10	80	3	13	22	50	61
Median of those with detectable cytokine		1·1	1·7	2·0	6·0	1·1	1·1	1·4
Median overall	–	–	1·0	–	–	–	1·5	0·9

(d) IL-5 – high intensity of infection

	No stimulant		AWA		No stimulant		PHA & LPS	
	Child	Adult	Child	Adult	Child	Adult	Child	Adult
N	23	5	23	6	30	8	30	8
Percentage with detectable cytokine	26	0	35	67	17	0	57	50
Median of those with detectable cytokine	2·2		1·8	1·6	2·9		1·6	4·4
Median overall	0·3	–	0·3	1·1	–	–	0·9	0·9

Table 1 *continued*
(e) IL-10 – low intensity of infection

	No stimulant		AWA		No stimulant		PHA & LPS	
	Child	Adult	Child	Adult	Child	Adult	Child	Adult
N	8	23	8	23	5	30	5	30
Percentage with detectable cytokine	63	52	88	56	40	27	100	100
Median of those with detectable cytokine	0·2	0·3	0·2	0·2	0·5	0·5	6·9	4·6
Median overall	0·04	0·08	0·2	0·04	0·04	0·04	6·9	4·6

(f) IL-10 – high intensity of infection

	No stimulant		AWA		No stimulant		PHA & LPS	
	Child	Adult	Child	Adult	Child	Adult	Child	Adult
N	30	8	30	8	23	6	23	6
Percentage with detectable cytokine	63	63	93	88	17	33	100	100
Median of those with detectable cytokine	1·0	0·06	0·2	0·1	0·9	0·04	6·7	4·3
Median overall	0·04	0·02	0·1	0·1	–	0·04	6·7	4·3

(g) IFNγ – low intensity of infection

	No stimulant		AWA		PHA & LPS	
	Child	Adult	Child	Adult	Child	Adult
N	8	23	8	23	8	23
Percentage with detectable cytokine	25	35	25	26	100	100
Median of those with detectable cytokine	2·9	6·3	16·9	3·9	429	622
Median overall	–	–	–	–	429	622

(h) IFNγ – high intensity of infection

	No stimulant		AWA		PHA & LPS	
	Child	Adult	Child	Adult	Child	Adult
N	30	8	30	8	30	8
Percentage with detectable cytokine	10	10	17	25	100	100
Median of those with detectable cytokine	3·5	4·8	6·1	15·2	230	301
Median overall	–	–	–	–	230	301

with a high intensity of infection). More IL-5 was produced by samples from children than adults with low intensities of infection, although the one sample of adult blood that produced IL-5 produced 2 IU/mL compared to the median of 1·7 IU/mL produced by blood from the children containing detectable cytokine. In the high intensity of infection category, median levels of IL-5 produced were higher in samples from adults than children, but median values calculated from samples producing detectable cytokine were slightly lower in adults than children (Table 1c,d). Concentration of IL-5 correlated negatively with age ($P < 0{\cdot}004$) if people had intensities of infection under 200 EPG ($P < 0{\cdot}05$ after Bonferroni correction), but correlated positively if they had over 400 EPG ($P < 0{\cdot}006$, $P = 0{\cdot}08$ after Bonferroni correction).

IL-5 PHA and LPS

There was no discernible pattern to IL-5 produced by samples cultured with PHA and LPs, and no statistically significant correlations with age or differences between groups. The median levels overall were higher in children (1·5 IU/mL) compared to adults (0·9 IU/mL) with low intensities of infection, but exactly the same in children and adults with high intensities of infection (0·9 IU/mL). (Table 1c,d). Median IL-

5 calculated only from samples producing detectable cytokine was higher in adults than children in both intensity of infection categories. A median of 1·4 IU/mL IL-5 was produced by adults and 1·1 IU/mL produced by children with low intensities of infection and a median of 4·4 IU/mL was produced by adults and 1·6 IU/mL produced by children with low intensities of infection. IL-5 produced by cultures stimulated with PHA and LPS did not correlate with age.

IL-10 AWA

Similar amounts of IL-10 were produced by samples cultured with AWA and unstimulated samples.

In AWA-stimulated cultures, in the low intensity of infection category, 88% of blood samples from children produced IL-10 compared to 56% of blood samples from adults. In blood from subjects in the high intensity of infection category, 93% of samples from children and 88% of samples from adults produced detectable cytokine. This difference was significant overall ($P < 0{\cdot}009$), but not significant after Bonferroni correction ($P = 0{\cdot}06$). In the low intensity of infection category, the median values overall were five times higher in children (0·2 IU/mL) than in adults (0·04 IU/mL), but when only those samples that produced detectable cytokine were included, the amount of IL-10 produced was the same in the two groups (0·2 IU/mL). In the high intensity of infection category, the median values overall were the same in children and adults (0·1 IU/mL), but in those samples that produced detectable cytokine, the amount of IL-10 produced was slightly higher in children (0·2 IU/mL) than adults (0·1 IU/mL). There was no significant correlation between IL-10 and age when samples were cultured with AWA.

IL-10 PHA/LPS

All samples produced IL-10. In both intensity of infection categories, samples from children produced more IL-10 (median = 6·9 IU/mL, low intensity of infection, 6·7 IU/mL high intensity of infection) than adults (median = 4·6 IU/mL low intensity of infection, 4·3 IU/mL high intensity of infection). There appears to be a tendency for the samples that produced over 5 IU/mL of IL-10 to be from younger people (Figure 1) but there was no significant correlation between age and IL-10 in either intensity of infection category after Bonferroni correction. Before correction, there was a significant correlation between age and IL-10 production in the high intensity of infection category ($P < 0{\cdot}02$).

IFNγ AWA

The percentages of samples producing IFNγ when cultured with AWA were similar in all groups (low intensity of infection: 25% children, 26% adults; high intensity of infection: 17% children, 25% adults). Median IFNγ calculated only from samples producing detectable IFNγ were higher in children compared to adults in the low intensity of infection categories but higher in adults compared to children in the high intensity of infection categories (Table 1g,h). IFNγ concentration did not correlate with age in either the low or high intensity of infection category.

IFNγ PHA and LPS

All samples produced IFNγ when cultured with PHA and LPS. The median levels were higher in adults than in children in both intensity of infection categories, but there was no statistically significant correlation between IFNγ concentration and age.

DISCUSSION

Children and adults tended to respond differently to schistosome antigen given equivalent exposure, number of years' experience and current levels of infection. The most significant illustration of this phenomenon was the negative correlation between age and IL-5 produced by samples from people with low intensities of infection cultured with AWA. The results also extend previous findings from an endemic area that IL-5 produced by antigen-stimulated cultures was positively correlated with age in individuals selected to have high intensities of infection (15) but indicate that the reverse could occur when subjects have lower intensities of infection.

IL-10 produced by samples cultured with PHA and LPS was notably lower in children than adults, although not formally significant. The analyses of the high intensity groups may have suffered from lack of power because of the low number of subjects in the group of adults with a high intensity infection. The difference between adults and children in the percentages of samples producing IL-10 in response to AWA was only evident when the more sensitive of the two ELISA protocols was used and the levels of IL-10 produced were in the same range as spontaneously released IL-10, indicating very low levels of antigen-specific IL-10 production. The tendency for samples from children to produce more IL-10 than samples from adults was magnified when blood was non-specifically stimulated with PHS and LPS. Another study in a 7-year-old focus of *S. mansoni* infection in the same area found no detectable IL-10 in antigen-stimulated cultures (12). Our results largely agree with the previous study in the same area in which spontaneous and mitogen-induced IL-10 production was associated with childhood with the caveat that infection intensity was also different between child and adult groups in that study (12). IL-10 production is characteristic of murine schistosomiasis (16),

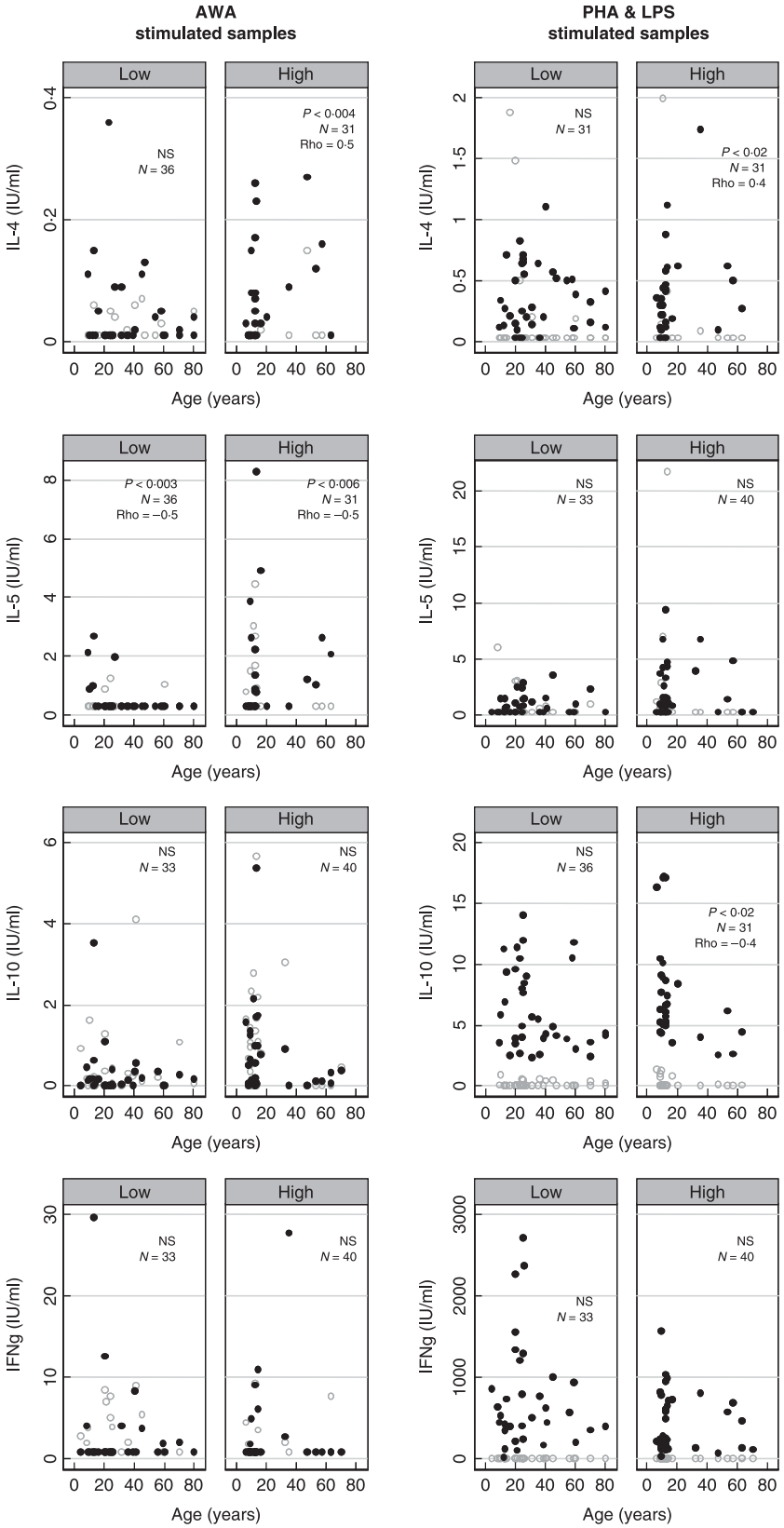

Figure 1 IL-4, IL-5, IL-10 and IFNγ produced by samples cultured with AWA and PHS and LPS (at peak of cytokine production) plotted against age. The significance of Spearman's rank correlations before Bonferroni correction (including samples that contained less than detectable cytokine) are noted. NS indicates not significant. Only IL-5 from people with low intensities of infection cultured with AWA correlated negatively with age after Bonferroni correction ($P < 0{\cdot}05$). Closed circles represent cytokine produced by stimulated cultures, open circles represent cytokine produced by the equivalent un-stimulated sample. 'Low' refers to subjects with intensities of infection less than 200 EPG. 'High' refers to subjects with intensities of infection more than 400 EPG.

is linked to protection against severe pathology in humans (17) and has been shown to down-regulate schistosome antigen-specific responses in several studies (17–21). Although considered a down-regulator of type-2 murine responses (16–22), human IL-10 can down-regulate both type 1 and type 2 T-cell responses (via inhibition of CD28 tyrosine phosphorylation (23,24)) and monocytes and macrophages (by other mechanisms (25)).

IL-4 correlated positively with age when samples were stimulated with antigen or mitogens. To our knowledge, IL-4 responses have not previously been associated with age. With the caveat that none of the results remained significant after Bonferroni correction, these data suggest that adults have a propensity to produce more IL-4 than children when they have similarly high intensities of infection.

Our results confirm and extend studies showing that age can be associated with the humoral immune response, independently of intensity of infection in endemic (26) and recently exposed (27,28) communities; age-related changes in cellular immune responses to schistosomiasis have also been reported in rhesus monkeys (29). This indicates that it is possible for age, independently of intensity of infection or experience of the parasite, to influence the type of immune response and therefore, potentially, the degree of protection afforded. This does not preclude the possibility of a concurrent role for experience of infection.

Our work in this recently established focus has indicated, controversially, that age-dependent factors are the main cause of resistance to *S. mansoni* infection, with a less marked role for resistance acquired over years of exposure (2). The current results indicate that there are differences in the manner in which an adult cellular immune system responds to schistosome antigens and non-specific mitogens. We previously speculated that intensity of infection in children reflects the transmission dynamics of their village, whereas variation between adult groups is limited by adult-specific protective factors (2). The current data cannot be used to indicate which types of immune response are protective because we have not correlated cytokine levels with infection levels after a period of re-infection. However, we can now further speculate that children and adults do have different capacities to mount a response to schistosome infection and adult protection may be the freedom to respond to different levels of infection in an appropriate way.

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