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Cellular cytotoxicity to measles virus during natural measles infection

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SUMMARY

Little is known about cellular cytotoxicity to measles virus during natural measles infection which is still a major cause of death in many parts of the world. Therefore we measured the ability of peripheral blood mononuclear cells (PBM) from children with measles to kill Hela cells persistently infected with measles virus. In a 6-hr ^{51}CR-release assay antibody-independent cellular cytotoxicity was shown to be low during the acute stage of measles. This rose to a maximum 1 week after the onset of the rash and fell rapidly on recovery 2 to 3 weeks later. The respective means values for the three periods (expressed as specific immune release of ^{51}Cr) were $7{\cdot}9 \pm 8{\cdot}4\%$, $31{\cdot}0 \pm 16{\cdot}4\%$ and $6{\cdot}1 \pm 7{\cdot}7\%$. Killing in this assay was not effected by T lymphocytes, for concentration of these cells by three different methods failed to increase cytotoxic power. In contrast peripheral blood mononuclear cells depleted of T lymphocytes showed greatly increased antibody-independent cellular cytotoxicity. Antibody-dependent cellular cytotoxicity was not found to vary significantly with the stage of measles. The mean values were $30{\cdot}0 \pm 13{\cdot}6\%$, $26{\cdot}6 \pm 11{\cdot}7\%$ and $23{\cdot}9 \pm 12{\cdot}1\%$ for the periods 0–2, 3–14 and 15–30 days after the onset of the rash. Both antibody-independent and antibody-dependent cellular cytotoxicity of PBM were lowered by layering these cells on immune complexes fixed to plastic or by incubating them with normal rabbit γ-globulin. Antibody-dependent cellular cytotoxicity was also lowered in the presence of 10% acute-phase autologous plasma. We concluded that antibody-independent cytoxocity was effected either by natural killer cells or by K cells using traces of antibody present in the assay. Antibody-dependent cellular cytotoxicity which is due to K cells may be modulated by circulating immune complexes during the course of disease.

INTRODUCTION

There is a traditional belief that measles infection is primarily controlled by cell-mediated immune reactions and that thymus-derived lymphocytes (T lymphocytes) effect this control (Burnet, 1968). The evidence hinges on the clinical observations that children with thymolymphatic deficiency (Nahmias *et al.*, 1967) or leukaemia (Enders *et al.*, 1959) often have severe fatal measles characterized by persistent viral infection without the typical rash whereas the disease runs its normal course in children with hypogammaglobulinaemia (Good & Zak, 1956). Experiments with monkeys also suggest that T lymphocytes control the course of natural measles since animals which are immunosuppressed with anti-thymocyte globulin clear virus slowly and produce antibody slowly (Hicks, Sullivan & Albrecht, 1977).

T lymphocytes are known to possess receptors for measles virus and kill measles-infected cells

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in an antibody-independent cellular cytotoxicity (AICC) test (Valdimarsson, Agnarsdottir & Lachmann, 1975). Extension of this work showed that T lymphocytes from immune adults purified by rosetting with IgG-coated ox red blood cells killed Hela cells persistently infected with measles virus. Killing was not HLA-restricted, for AICC was independent of shared HLA antigens between target and effector cells (Ewan & Lachmann, 1977). Later Wright & Levy (1979) in a complicated series of experiments demonstrated HLA-restricted killer cells by incubating peripheral blood mononuclear cells (PBM) from immune adults on fibroblasts infected with measles virus. Two different types of cytotoxic lymphocytes were generated: one, a T cell, was fully HLA-restricted, and the other, which was not a T cell, showed an unusual form of HLA-restricted cytotoxicity. It lysed infected target cells autologous to either the PBM or the sensitizing monolayer but not third-party target cells allogeneic to both.

On the other hand, Perrin, Tishon & Oldstone (1977) could not demonstrate AICC of measles-infected cells by T lymphocytes of adult donors. They concluded that antibody-independent killing was probably effected by K cells either using traces of antibody carried cytophilically on PBM or antibody produced *in vitro* by lymphocytes contacting measles virus antigens. These authors also demonstrated that antibody-dependent cellular cytotoxicity (ADCC) of measles-infected cells was more efficient than antibody-independent cytotoxicity requiring a much lower effector: target cell ratio and less time. Ault & Wiener (1979) have shown that peripheral blood mononuclear cells (PBM) from immune adults contain natural killer cells which kill Hela cells infected with measles virus as well as the myeloid cells K562. These cells did not require antibody for their action, possessed Fc receptors for IgG and were shown to be neither T nor B cells.

Few measurements of cellular cytotoxicity have been made in patients with natural measles. Chiba, Yamanaka & Nakao (1974) using a long incubation AICC assay detected cell-mediated immunity to measles in five children 4 to 10 days after the rash. Kreth, ter Meulen & Eckert (1979) have demonstrated that AICC to measles virus at the time of the rash is more efficient if HLA antigens are shared between effector and target lymphocytes. These effector cells may have been T lymphocytes, but insufficient data was presented to draw a firm conclusion. However, these authors and others (Perrin *et al.*, 1978) have been unable to show either cytotoxic T cells or HLA restriction of killing following vaccination with measles virus. We are unaware of any reports of ADCC during natural measles infection.

In the hope of acquiring more knowledge about the mechanism of immunity to measles which is a major killing disease in Africa (Morley, 1969), we have studied antibody-independent and antibody-dependent cellular cytotoxicity to measles in children at different stages during the first month of measles. We have also attempted to define which subset of lymphocytes is responsible for the two types of cytotoxic mechanism.

MATERIALS AND METHODS

Patients. Children with measles diagnosed by the presence of Koplik's spots and/or a typical rash were studied as out-patients at the Ahmadu Bello University Hospital, Zaria, Kaduna State, Nigeria at various intervals after the onset of the rash. Only well-nourished children (weight for age greater than 80% of Harvard Standard) were selected. The children were classified as having had mild, moderate or severe disease according to the criteria of Scheifele & Forbes (1972). The mean age of patient was 17·4 months (range 9–48 months). The study was approved by the Ethical Committee of the Faculty of Medicine, Ahmadu Bello University.

Separation and identification of peripheral blood mononuclear cells. Ten millilitres of venous blood were collected in 100 iu of preservative-free heparin and the PBM separated by density-gradient centrifugation on Ficoll–sodium metrizoate as previously described (Whittle *et al.*, 1978). Our methods for identifying T lymphocytes by rosetting with sheep red cells (SRBC) and for identifying B lymphocytes by staining with fluorescein isothiocyanate-labelled sheep anti-human immunoglobulin have also been described in the above paper.

Separation of T lymphocytes. Three methods were used:

(i) *Nylon fibre.* PBM were passaged through a nylon-fibre column yielding over 90% T lymphocytes, less than 2% B lymphocytes and a variable proportion of non-rosetting lymphocytes which did not stain with fluorescein-labelled anti-human immunoglobulin. These are termed 'null cells'.

(ii) *Sheep red blood cell rosettes.* PBM were mixed with 2% SRBC in the presence of 40% heat-inactivated foetal calf serum (FCS) which had previously been absorbed with SRBC. The cells were incubated for 5 min at 37°C, spun at 200 *g* for 7 min at 4°C and held at 4°C for 2 hr in crushed ice. They were then gently mixed with a Pasteur pipette, layered onto cold Ficoll–sodium metrizoate and centrifuged at 400 *g* for 30 min at 4°C. The cell pellet contained over 90% erythrocyte rosette-positive cells ($SRBC^+$) and less than 2% B lymphocytes.

The layer of PBM above the Ficoll consisted of B lymphocytes, macrophages and null cells. It contained less than 2% $SRBC^+$ cells.

(iii) *Ox red blood cell rosettes.* Antibody to ox red blood cells (ORBC) was raised in rabbits by standard methods. The IgG fraction of this sera was obtained by separation on diethylaminoethyl cellulose. Five per cent washed fresh ORBC were suspended in minimum essential medium (MEM) and 1% normal rabbit serum. This serum had previously been absorbed with ORBC. An equal volume of anti-ORBC IgG (titre of anti-ORBC antibodies 1 : 64) was added to the cells and kept at 20°C for 30 min. The cells were then washed in MEM three times and made up to 2% in MEM containing 1% bovine serum albumin. Five million PBM in 0·5 ml MEM were added to an equal volume of the IgG-coated ORBC in 12 × 75 mm tissue culture tubes. The tubes were spun at 200 *g* for 5 min and kept at 4°C for 1 hr. The cells were then gently mixed with a Pasteur pipette and separated by density-gradient centrifugation on Ficoll–sodium metrizoate at 20°C. The top layer of cells which was used for the cytotoxicity tests consisted of lymphocytes, 90% of which rosetted with SRBC.

Handling and infection of Hela cells. Hela cells obtained from the National Institute of Biological Standards, Holly Hill, London were persistently infected with wild-type Edmonston strain measles virus (kindly donated by Dr E. McIntyre, National Institute for Medical Research, Mill Hill, London) according to the method of Joseph & Oldstone (1975). Briefly, Hela cells were grown to a monolayer in 75-cm^2 plastic tissue culture flasks in MEM supplemented with 10% FCS, glutamine, 100 iu/ml penicillin, 100 μg/ml streptomycin and 2·5 μg/ml amphotericin B. The medium was buffered with 20 mmol/l Hepes and 10 mmol/l sodium hydroxide. Cells were removed with EDTA/0·05% trypsin, suspended in growth medium in 100 × 16 mm tissue culture tubes at 1×10^6/ml and infected with measles virus at a multiplicity of infection of 0·1. After 1 hr the cells were washed and transferred to 75-cm^2 tissue culture flasks in growth media and incubated at 37°C. The media in these flasks was changed twice per week and after 6–8 weeks a confluent monolayer of infected cells was obtained. These cultures were divided weekly as were the uninfected Hela cells. The viability of the persistently infected cells was >95% as judged by exclusion of trypan blue and over 95% of these cells expressed measles virus on their surface as judged by immunofluorescent staining.

Tests of cell-mediated cytotoxicity. Measles-infected and uninfected control cells were grown for 3 days, the monolayers were then removed with EDTA/0·05% trypsin and washed once in cold MEM containing 1% FCS by gentle centrifugation at 200 *g* for 7 min at 4°C. Two million of the cells were suspended in 0·5 ml Tris/HCl buffer, pH 7·4, containing 10% FCS in 12 × 75 mm tissue culture tubes. One hundred microcuries of sodium ^{51}Cr chromate in sodium chloride was added to each tube, incubated at 37°C for 1 hr and then washed three times in cold MEM/1% FCS. After checking the viability of the cells (average >90% viable) the ^{51}Cr-labelled cells were suspended at 1×10^5 cells/ml in Roswell Park Memorial Institute medium (RPMI 1640) which was buffered with 20 mmol/l Hepes and 10 mmol/l NaOH and contained antibiotics but no amphotericin.

PBM were suspended at $3{\cdot}5 \times 10^6$/ml in the above medium for the AICC test and at $1{\cdot}25 \times 10^6$/ml for the ADCC test. Using an Eppendorf pipette, 0·1 ml of these cell suspensions were dispensed into each of five wells of a *U* microtitre plate. Target cells (0·05 ml) were then added to each well and 0·05 ml of 40% FCS or 40% FCS plus 2% pooled measles convalescent serum was added for the AICC or ADCC tests respectively. Thus for the AICC test an effective

target cell ratio of 70:1 was used in a final volume of 0·2 ml medium containing 10% FCS. For the ADCC test, the effective target ratio was 23:1 in medium containing 10% FCS with 0·5% measles convalescent serum. This pool of serum obtained from children 1 month after measles contained measles haemagglutination-inhibiting antibody at a titre of 1:1,012. Preliminary tests of ADCC showed that a final concentration of 0·5% of this serum was the minimal concentration that gave optimal lysis of target cells. In some ADCC tests a final concentration of 10% autologous plasma (heated at 56°C for ½ hr) plus 0·5% measles convalescent serum was used instead of FCS and antibody. Spontaneous release of ^{51}Cr from measles-infected and normal Hela cells was assessed by incubating these cells in media containing 10% of the appropriate sera but no PBM. Maximum release of ^{51}Cr was determined by adding Triton X detergent at a final concentration of 2·5% to the labelled cells.

The microtitre plates were sealed with plastic covers, spun at 200*g* for 7 min and incubated at 37°C for 6 hr. The plates were again centrifuged and 0·1 ml of supernatant removed from each well with an Eppendorf pipette. The aliquots were pooled in glass vials and counted in a gamma counter for 180 sec. Cytotoxicity expressed as specific immune release (SIR) of ^{51}Cr was calculated by the formula:

$$\text{Percentage SIR} = \frac{(M-Ms)}{(\text{Max } M-Ms)} - \frac{(C-Cs)}{(\text{Max } C-Cs)} \times 100,$$

where M or C = ^{51}Cr (in c.p.m. − background count) released from measles-infected Hela cells or uninfected Hela cells respectively in the presence of PBM; Ms or Cs = ^{51}Cr (in c.p.m. − background count) released spontaneously from infected or uninfected Hela cells and Max M and Max C = ^{51}Cr (in c.p.m. − background count) released by 2·5% Triton X from infected and uninfected Hela cells. In fifteen experiments the spontaneous release of ^{51}Cr averaged 10·8±3·6% and 7·1±1·7% of the total radioactivity incorporated into the measles-infected and uninfected Hela cells respectively. The maximum release of ^{51}Cr from measles and control cells was 59·0±12·7% and 57·0±9·8% of the total.

Preliminary experiments using $SRBC^+$ lymphocytes showed that lysis of SRBC by ammonium chloride destroyed AICC. These experiments also showed that the addition of the equivalent number of SRBC as that used in the rosette separation procedure did not change the AICC values of PBM. Thus AICC tests of $SRBC^+$ lymphocytes were measured in the presence of SRBC.

In other preliminary experiments PBM were mixed with uncoated ORBC, kept at 4°C for 1 hr, separated by buoyant density centrifugation and used in the AICC test. This procedure did not alter AICC values.

Absorption of PBM of plastic coated with antigen–antibody complexes. One-half a millilitre of a solution of crystalline human serum albumin at a concentration of 6 mg/ml in phosphate-buffered saline (PBS), pH 7·2, was pipetted into 16-mm plastic multidisk culture trays. After 30 min at 20°C the dishes were washed three times with PBS and then incubated with 0·5 ml of either PBS or monospecific rabbit anti-HSA (Hoechst Pharmaceuticals) which was diluted 1:10 in PBS. After 30 min at 20°C the plates were washed with PBS and 1 ml of PBM at 1×10^6/ml in RPMI/10% FCS was pipetted into each well. After 90 min at 37°C the plates were gently swirled for 5 min and the non-adherent cells removed with a Pasteur pipette. These were washed in MEM/1% FCS and made up to the appropriate concentration in RPMI for use in the AICC and ADCC tests.

Incubation of PBM with normal rabbit gammaglobulin. Normal rabbit gammaglobulin was prepared from normal rabbit serum by precipitation with 27% sodium sulphate. After exhaustive dialysis again in PBS the globulin was diluted 1 in 5 in RPMI and 0·5 ml of this preparation was incubated for 30 min at 37°C with 5×10^6 PBM. These cells were then washed three times in MEM/1% FCS, adjusted to the appropriate concentration in RPMI, and used for the cytotoxicity tests.

Measurement of measles antibody. Measles haemagglutination-inhibiting antibody was measured in *V*-well microtitre plates using Tween–ether-treated measles haemagglutinin (Hoechst Pharmaceuticals) as the antigen and green monkey red blood cells as the indicator.

Results are expressed in units which are $\log_2$ of reciprocal of the titre of serum which gave complete inhibition of haemagglutination.

Statistics. Means are expressed as ±1 standard deviation. Student's *t*-test was used to test the significance of the difference between the means. A *P* value of less than 0·05 was regarded as significant.

RESULTS

Antibody-independent cellular cytotoxicity. Fig. 1 shows that the AICC of children with measles rose and fell significantly ($P<0{\cdot}001$) during the first month of measles. The respective means were 7·9±8·4%, 31·0±16·4% and 6·1±7·7% for the periods 0–2, 3–14 and 15–30 days after the onset of the rash. Killing of uninfected cells, which averaged 15·8±12·1%, did not vary significantly over these periods. Mononuclear cells from four adults and from two cord bloods were also tested in the AICC assay. Values for the four adults averaged 5·1%; no cytotoxicity by the cord blood lymphocytes could be demonstrated.

Relation to serum antibody levels. The mean measles haemagglutination-inhibiting antibody levels for the three periods were 2·1±2·2, 6·9±2·3 and 7·3±2·3 units respectively. During the acute stage (days 0–2) the two children with the highest level of AICC also had the highest levels of antibody in their serum (6 units each).

Clinical correlates. There was no obvious correlation between the severity of measles and the degree of AICC in any of the three stages of the disease. In the second stage the mean of the seven children with mild measles was 36·4±16·8% as compared to a mean of 26·2±15·4% for the eight children with moderate or severe measles (*P* not significant).

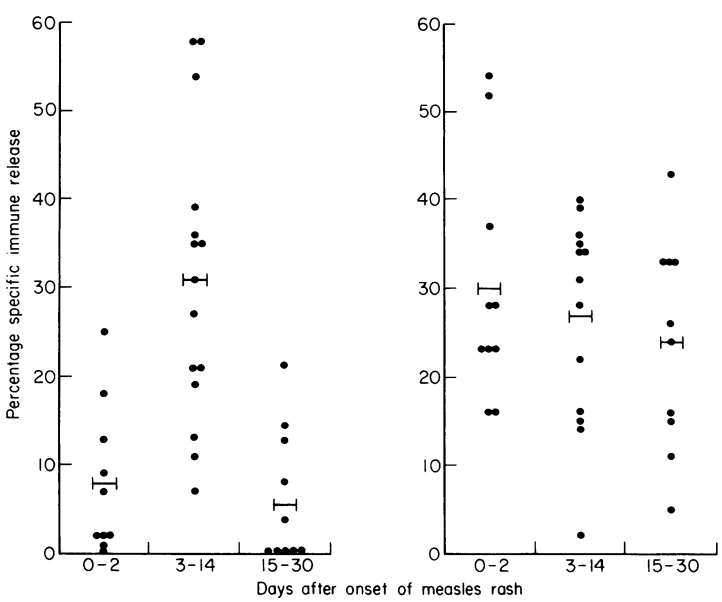

Fig. 1. Antibody-independent cellular cytotoxicity: percentage specific immune release of ^{51}Cr from Hela cells persistently infected with measles virus by PBM from children at various intervals after the onset of measles rash. Bar indicates mean.

Fig. 2. Antibody-dependent cellular cytotoxicity: percentage specific immune release of ^{51}Cr from Hela cells persistently infected with measles virus by PBM from children at various intervals after the onset of measles rash. Bar indicates mean.

Effect of different cell fractions on AICC. Table 1 shows the effect of different cell fractionation procedures on AICC. The PBM were obtained from children on average 5 days after the onset of the measles rash. Enrichment with T cells by any of the three methods employed did not increase the AICC whereas depletion of T cells markedly increased AICC.

Effect of normal rabbit gammaglobulin and bound immune complexes on AICC. Table 2 shows that prior incubation of PBM with normal rabbit gammaglobulin lowers the AICC values as does incubation on a plastic surface coated with HSA and anti-HSA complexes.

Antibody-dependent cellular cytotoxicity. The percentage specific immune release of ^{51}Cr in the presence of antibody from measles-infected Hela cells by PBM from children at different stages of measles is shown in Fig. 2. The respective mean values were 30·0±13·6%, 2·6±11·7% and 23·9±12·1% for the periods 0–2, 3–14 and 15–30 days after the onset of the rash. The differences are not statistically significant.

Table 1. Effect of different purification procedures on antibody-independent cytotoxicity of lymphocytes from children with measles*

		Purification procedure			
				SRBC rosettes	
Patient	PBM (unfractionated)	Nylon column T-enriched	Ox RBC rosettes T-enriched	Positive T-enriched	Negative T-depleted
A	45	37	—	—	—
B	14	17	—	—	—
C	24	—	3	—	—
D	21	—	0	—	—
E	36	—	6	—	—
F	—	—	—	14	50
G	16	—	—	16	53
H	17	—	—	23	61

* All values expressed as percentage specific immune release of ^{51}Cr from Hela cells persistently infected with measles virus.

Table 2. The effect of normal gammaglobulin or bound immune complexes on antibody-independent and antibody-dependent cytotoxicity of peripheral blood mononuclear cells from children with measles*

	Antibody-independent cytotoxicity			Antibody-dependent cytotoxicity		
		PBM			PBM	
Patient	PBM alone	+ Gammaglobulin	+ Immune complexes	PBM alone	+ Gammaglobulin	+ Immune complexes
I	48	37	—	16	12	—
J	30	15	—	18	9	—
K	17	—	3	42	—	9
L	16	—	1	14	—	0

* All values expressed as percentage specific immune release of ^{51}Cr from Hela cells persistently infected with measles virus.

Table 3. Effect of autologous plasma* on antibody-dependent cellular cytotoxicity of peripheral blood mononuclear cells from children during measles

Stage of disease	Per cent specific immune release ^{51}Cr		
	FCS + Ab	Plasma + Ab	Per cent inhibition†
Acute ($n=8$)	30±13	8±8	72·0±24
Convalescent ($n=8$)	24±12	15±10	44±25

* Taken at the time cells were collected.

† Per cent inhibition $= 1 - \frac{\text{ADCC in plasma}}{\text{ADCC in FCS}} \times 100.$

Effect of autologous plasma on ADCC. Table 3 shows the effect of 10% autologous plasma plus 0·5% antibody on the ADCC of PBM from children during the acute stage of measles (0–2 days after onset of rash) and from convalescent children (15–30 days after the start of the rash). Autologous plasma inhibits ADCC and this inhibition is significantly greater in the acute stage of measles than on recovery ($P<0·05$).

Effect of normal rabbit gammaglobulin and bound immune complexes on ADCC. Table 2 shows that incubation of PBM with normal rabbit gammaglobulin or with HSA–anti-HSA complexes fixed to plastic lowers the ADCC of these cells. The PBM were obtained from children on average 5 days after the onset of the measles rash.

DISCUSSION

This study shows that AICC is a transient phenomenon during measles rising to a maximum 7 days after the onset of the rash and falling rapidly during the next 2 or 3 weeks. In this respect measles resembles a number of experimental viral infections such as vaccinia infection in mice where cytotoxic T cells are active for a limited time after infection (Zinkernagel & Althage, 1977). This transient response may explain why other scientists have had difficulty in demonstrating AICC to measles in adults since low values are obtained only at high effector to target cell ratios after a long incubation. AICC in our assay was mediated by lymphocytes which are not T cells. We state this because enrichment of PBM with T cells failed to enhance AICC and depletion of T cells markedly increased cytotoxicity. Killing power of PBM was unaltered when macrophages were removed by passage through a nylon column but was lowered by rabbit γ-globulin or bound immune complexes. Thus our evidence points to a cell with Fc receptors as the mediator of AICC after measles. This may be a natural killer cell perhaps activated to maximum efficacy 1 or 2 weeks after the appearance of the rash by interferon (Herberman, Ortaldo & Bonnard, 1979). Alternatively, it may be a K cell using antibody produced *in vitro* by B cells. The rise and fall of cytotoxicity could be explained by variation in the ability of B cells to produce antibody *in vitro* at different stages of the disease. Further experiments showing that $F(ab')_2$ anti-IgG fragments could block killing and that killing is limited to measles and not other virus-infected cells would be needed to resolve this issue.

In contrast to AICC, ADCC is maximal at the onset of the rash and falls slightly but insignificantly during the disease. Autologous plasma inhibits this type of killing, especially during the acute stage of the disease, presumably because circulating immune complexes bind to the Fc receptors of K cells. We were also able to lower ADCC by blocking Fc receptors with gammaglobulin or by removing cells bearing such receptors by absorption with bound immune complexes. The role that circulating immune complexes have in controlling ADCC *in vivo* has not been thoroughly explored. The ratio of free to antigen-bound antibody is likely to be important in the regulation of this allergic mechanism as Melewicz *et al.* (1977) have shown that herpes

antigen–antibody complexes block ADCC more effectively when low concentrations of antibody are used in the test. They suggested that this inhibitory mechanism may prevent the host from autoimmune attack against cells which bear cross-reactive antigens.

We would like to make it clear that our assay of antibody-independent cytotoxicity was not suitable for the measurement of T cell killing for we were unable to HLA-match target cells to patients' lymphocytes. In a few preliminary experiments we could not demonstrate AICC to autologous lymphocytes which had been separated at the start of the rash and stimulated with PHA. At this time a proportion of stimulated lymphocytes normally express measles virus on their surface and are susceptible to lysis by antibody and complement (Whittle *et al.*, 1978). Perhaps T cell cytotoxicity had already waned by then for killing by these cells is often transient and appears early in viral infections (Zinkernagel, 1978). At present the question as to whether HLA-restricted T cell killing occurs in natural measles remains unanswered.

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