*Clin. exp. Immunol.* (1977) **29**, 11–15.

# Antibody-dependent cellular cytotoxicity (ADCC) against Epstein–Barr virus-determined antigens

## III. REACTIVITY IN SERA FROM PATIENTS WITH BURKITT'S LYMPHOMA IN RELATION TO TUMOUR DEVELOPMENT

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(*Received* 6 *January* 1977)

SUMMARY

Sera from patients with Burkitt's lymphoma (BL) were tested for antibody-dependent cellular cytotoxicity (ADCC) against Epstein–Barr virus (EBV) determined antigens and for antibodies directed against various EBV-specific antigens. No correlation was found between EBV serology and ADCC in sera selected for high or low titres against the membrane antigens (MA) or in consecutive sera from three patients with isolated late tumour recurrences. Furthermore, no correlation was found between ADCC and tumour development.

## INTRODUCTION

The Epstein–Barr virus (EBV) is considered to play some role in the development of BL (Klein, 1975a). EBV DNA and an EBV-specific nuclear antigen, EBNA, is present in more than 95% of BL tumour biopsies (Klein, 1975b).

EBV is a ubiquitious virus and seropositive individuals have antibodies which induce ADCC against cells that express EBV-related antigens (Jondal, 1976; Pearson & Orr, 1975). It is possible that EBV-specific ADCC acts, together with other immunological effector mechanisms, to maintain the virus in a latent state. Whether ADCC influences tumour growth in BL patients is unclear.

In the present paper we have studied the capacity of BL sera, harvested at different stages of tumour development, to induce specific ADCC and compared this reactivity with the titres of antibodies directed against EBV-specific membrane antigens (MA), viral capsid antigens (VCA) and early antigens (EA). ADCC has been tested by a procedure which earlier has been found optimal for the EBV system (Jondal, 1977).

## MATERIALS AND METHODS

*Lymphocytes.* Aggressor lymphocytes were purified by centrifugation of heparinized whole blood on Ficoll–Isopaque, phagocytic cells were removed by the iron carbonyl powder technique (Jondal, 1974). Lymphocytes were taken from normal healthy individuals either inside or outside the laboratory.

*Target cell.* Daudi cells, derived from Burkitt's lymphoma tumour cells (Klein *et al.*, 1968), were used as target cells. These cells express binding sites for Fc, C3 and EBV and lack detectable amounts of $B_2$-microglobulin and HLA (Klein *et al.*, 1976). Furthermore, Daudi cells express a high amount of surface-bound IgM (Klein *et al.*, 1968). Daudi cells were propagated as a suspension culture in RPMI with 10% of foetal calf serum and antibiotics.

*Sera.* Sera from patients with African Burkitt's lymphoma were heat-inactivated (56°C for 30 min) upon arrival to the department and stored at −20°C. Chains of sera, harvested from the same patient at different time points, were thawed and tested simultaneously.

*ADCC assay.* The ADCC assay was done as described and analysed in detail elsewhere (Jondal, 1976). Sera, 25 μl, was mixed with 25 μl of EBV, i.e. supernatants from the virus-producing B-95-8 cell line (Miller *et al.*, 1972). Allogeneic lympho-

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cytes, 50 $\mu$l, and 50 $\mu$l of $^{51}$Cr-labelled Daudi cells in V-shaped microplates. Lymphocytes, $2\times10^5$, were used with $10^4$ target cells giving an aggressor: target ratio of 20:1. After 3–5 hr 75 $\mu$l of supernatant was harvested and cytotoxicity calculated according to the formula:

$$\frac{\text{test release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100.$$

ADCC was performed in RPMI 1640 supplemented with antibiotics and 5% foetal calf serum.

*Assay of antibodies to MA.* Undiluted sera or test serum dilutions were used to block the direct membrane immunofluorescence reaction between MA-positive cultured lymphoblastoid cells and a reference anti-MA IgG conjugate from a BL patient (conjugate F-Mutua). A blocking index was calculated as the difference between the percentages of positive cells in an unblocked reference cell sample and in the blocked test sample, divided by the first percentage (Gunven & Klein, 1971). Mean indexes were recorded after at least three tests under code of each serum dilution. In titration, six two-fold dilution steps of each serum were tested on repeated occasions. The anti-MA titers of the sera, defined by a blocking index of 0·40, were calculated as described (Gunven & Klein, 1971).

*Assays of antibodies to EA and VCA.* Titres of these antibodies were kindly provided by Dr W. Henle and Dr G. Henle. The techniques employed for preparation of acetone- or methanol-fixed cell smears, the titration of sera in indirect immunofluorescence tests, and the reading of results have been described (Henle *et al.*, 1966, 1969, 1971b). For anti-VCA determinations, smears were prepared from P3 HR-1 cells which had been maintained on arginine-deficient medium for 3 days. For anti-EA determinations, smears were made from Raji cultures which had been inoculated 2 days previously with EBV derived from the P3 HR-1 cell line. The virus inoculum was adjusted to yield from 5–15% EA-positive cells, and no, or only rare, VCA-positive cells. Anti-EA was differentiated as to anti-D or anti-R reactivity on the basis of the staining patterns and reactivities with acetone- and/or methanol-fixed cell smears (Henle *et al.*, 1971b).

## RESULTS

### *ADCC in relation to EBV serology*

Patient sera were selected according to their anti-MA reactivity and tested for ADCC. In Table 1 an experiment including eleven sera is given. It is clear that there is no obvious correlation between ADCC reactivity and titres against MA, VCA and EA. In fact, the weakest serum in ADCC (Iraga)

TABLE 1. Patient sera were selected according to their anti-MA reactivity and tested for EBV serology and EBV-dependent ADCC

| Patient | | EBV dependent ADCC* | | | Controls without addition of EBV† | | | Month before death | EBV serology‡ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ($10^{-2}$ | $10^{-3}$ | $10^{-4}$)×6 | ($10^{-2}$ | $10^{-3}$ | $10^{-4}$)×6 | | MA | VCA | EA | |
| | | | | | | | | | | | R | D |
| 1166 | Rael Wafula | 49 | 30 | 21 | 16 | 18 | 18 | 13 | 4 | 160 | 40 | neg. |
| 1227 | Solubu Ngono | 36 | 52 | 30 | 21 | 20 | 21 | 11 | 5 | 640 | 20 | neg. |
| 1349 | Oduor Oloo | 50 | 51 | 28 | 27 | 22 | 18 | LTS | 20 | 1280 | 320 | 20 |
| 1084 | Niva Kayali | 27 | 32 | 23 | 16 | 18 | 16 | 1 | 20 | 640 | ? | 160 |
| 1199 | Halima Nabiba | 59 | 37 | 23 | 17 | 18 | 19 | LTS | 30 | 320 | 40 | neg. |
| 1315 | Naliaka Barasa | 48 | 30 | 19 | 21 | 20 | 21 | 7 | 45 | 160 | 80 | neg. |
| 1065 | Henry Jilonga | 49 | 49 | 28 | 19 | 19 | 18 | 5 | 70 | 320 | 160 | 20 |
| 1021 | Wawire Wafula | 30 | 51 | 29 | 23 | 22 | 20 | LTS | 90 | 320 | 320 | 40 |
| 976 | Namalwa Joseph | 46 | 35 | 20 | 19 | 23 | 21 | 10 | 110 | 640 | 640 | neg. |
| K-BCG | Amaba | 46 | 49 | 23 | 19 | 18 | 18 | 15 | 280 | 160 | neg. | neg. |
| K-BCG | Iraga | 36 | 23 | 18 | 20 | 20 | 20 | LTS | 450 | 320 | 10 | neg. |

LTS = Long term survival.

* EBV-dependent ADCC was tested at indicated serum dilutions.

† Control tests were performed in the presence of medium instead of EBV. Spontaneous lymphocyte-mediated cytotoxicity (SLMC) in the absence of patient serum was 19%.

‡ Anti-MA titres were defined by a blocking index of 0·40 as described in the Materials and Methods section. Anti-VCA and anti-EA titres were defined as end point dilutions giving positive fluorescence. Anti-EA titres are given against restricted (R) and diffuse antigens (D). When no distinction is done between R and D, values are indicated in the middle.

has the highest titre against MA. In Table 1, controls without addition of EBV are also given. It can be seen that the sera do not induce ADCC against antigens on Daudi cells which are unrelated to EBV. As several of the BL patients received blood transfusions, it is probable that some sera contain anti-HLA antibodies which could be expected to induce ADCC against cells with the relevant HLA haplotypes, however, in the present test system this was no problem as Daudi cells are known to lack HLA (Klein *et al.*, 1976).

Cytotoxicity in controls is thus mediated by normal lymphocytes alone [spontaneous lymphocyte-mediated cytotoxicity, SLMC (Jondal & Pross, 1975)], which also is true for experiments reported in Tables 2, 3 and 4). In these latter experiments, SLMC is subtracted from all values.

TABLE 2.

| Patient Salim Mwalim (date of serum harvest) | Specific EBV-dependent ADCC* | | | Clinical status at time of serum harvest | EBV serology† | | | |
|---|---|---|---|---|---|---|---|---|
| | $(10^{-2}$ | $10^{-3}$ | $10^{-4})\times 6$ | | MA (blocking index) | VCA | EA | |
| | | | | | | | R | D |
| 27-06-69 | 23 | 21 | 9 | Primary tumour | 4 | 40 | Negative | Negative |
| 19-09-69 | 23 | 18 | 5 | Recurrent tumour | 5 | 80 | Negative | Negative |
| 28-11-69 | 29 | 19 | 12 | Remission | 5 | 40 | Negative | Negative |
| 23-01-70 | 33 | 13 | 7 | Remission | 3 | 40 | Negative | Negative |
| 27-02-70 | 27 | 14 | 8 | Remission | 3 | 40 | Negative | Negative |
| 13-03-70 | 23 | 12 | 6 | Remission | 4 | 80 | Negative | Negative |
| 17-04-70 | 33 | 15 | 6 | Remission | 1·7 | 80 | Negative | Negative |
| 17-07-70 | 24 | 13 | 4 | Multiple recurrences | 2·3 | 160 | 20 | Negative |
| 04-09-70 | 29 | 20 | 6 | Regression | 5 | 640 | ? | 160 |
| 02-10-70 | 26 | 17 | 11 | Regression | 5 | 1280 | ? | 160 |
| 02 11 70 | 30 | 21 | 16 | Remission | 4 | 1280 | ? | 320 |
| 11-06-71 | 27 | 22 | 13 | Remission | 5 | 1280 | ? | 320 |

* Cytotoxicity in controls (as shown in Table 1) was subtracted from all values. SLMC in the absence of patient serum was 34%.

† See legend to Table 1.

TABLE 3.

| Patient Fanis Andawa (date of serum harvest) | Specific EBV-dependent ADCC* | | | Clinical status at time of serum harvest | EBV serology† | | | |
|---|---|---|---|---|---|---|---|---|
| | $(10^{-2}$ | $10^{-3}$ | $10^{-4})\times 6$ | | MA (blocking index) | VCA | EA | |
| | | | | | | | R | D |
| 31-07-66 | 8 | 17 | 7 | Remission | 0·52 | 160 | 40 | |
| 08-08-66 | 15 | 16 | 1 | Remission | 0·56 | 160 | n.d. | |
| 28-04-67 | 12 | 19 | 12 | Remission | 0·68 | 160 | 160 | Negative |
| 15-05-67 | 15 | 17 | 8 | Remission | 0·32 | 160 | n.d. | |
| 09-01-68 | 12 | 18 | 10 | Recurrence | 0·18 | 160 | 40 | |
| 12-01-68 | 12 | 12 | −1 | Recurrence | 0·41 | 40 | 80 | Negative |
| 08-03-68 | 16 | 13 | 7 | Recurrence | 0·70 | 160 | 80 | |
| 17-04-68 | 10 | 9 | 3 | Recurrence | 0·73 | 320 | 320 | 10 |
| 26-04-68 | 30 | 19 | 3 | Recurrence | 0·64 | 640 | 160 | |
| 17-05-68 | 23 | 20 | 3 | Recurrence | 0·58 | 320 | 320 | |

n.d. = Not done.

* See legend to Table 2. SLMC in the absence of patient serum was 21%.

† In this experiment anti-MA reactivity was only determined as the blocking index of undiluted patient serum. See also legend to Table 1.

*ADCC in relation to tumour development in individual patients*

In Tables 2, 3 and 4, consecutive sera from three patients with isolated late tumour recurrences were tested for ADCC which was related to the EBV-associated serology. All three patients had statistically significant drops of their anti-MA titres well in advance of their recurrences. Fanis and Salim had increases of their anti-VCA titres after the manifestations of their recurrences, and Salim also increased his anti-EA titres of both D and R specificity, markedly simultaneously with the appearance of recurrent tumours.

TABLE 4.

| Patient Ngui Shioki (date of serum harvest) | Specific EBV-dependent ADCC* | | | Clinical status at time of serum harvest | EBV serology† | | | |
|---|---|---|---|---|---|---|---|---|
| | ($10^-$ | $10^{-3}$ | $10^{-4}$)×6 | | MA | VCA | EA | |
| | | | | | | | R | D |
| 25-04-66 | 1 | 19 | 11 | Primary tumour | 50 | 640 | 160 | Negative |
| 25-07-66 | 3 | 7 | 15 | Remission | 50 | 640 | n.d. | n.d. |
| 30-12-66 | 7 | 15 | 18 | Remission | 35 | 320 | n.d. | n.d. |
| 06-02-67 | 5 | 16 | 18 | Remission | 50 | 320 | n.d. | n.d. |
| 17-04-67 | 6 | 21 | 23 | Remission | 45 | 320 | n.d. | n.d. |
| 08-05-67 | 15 | 26 | 27 | Remission | n.d. | 320 | n.d. | n.d. |
| 12-06-67 | 22 | 22 | 15 | Remission | 32 | 160 | n.d. | n.d. |
| 02-02-68 | 16 | 21 | 13 | Remission | 15 | 320 | 160 | |
| 21-06-68 | 12 | 27 | 21 | CNS recurrence | 15 | 640 | 80 | |
| 12-07-68 | 9 | 21 | 17 | CNS recurrence | 25 | 320 | 80 | Negative |
| 02-08-68 | 10 | 28 | 16 | CNS recurrence | 30 | 320 | 80 | Negative |
| 19-08-68 | 13 | 20 | 17 | CNS recurrence | 30 | 640 | 40 | |

n.d. = Not done.

* See legend to Table 2. SLMC in the absence of patient serum was 21%.

† See legend to Table 1.

It is seen that, as in Table 1, there is no apparent relationship between any of the conventional anti-EBV titres and the ADCC activity. Anti-MA titres range from very low (Fanis) to relatively high (Ngui) but this is not reflected in the ADCC titres. Also, anti-VCA and anti-EA titres have a considerable range between and within the patients but do not appreciably correlate with ADCC titres. The well-defined, very late tumour recurrences in these patients were unaccompanied by any changes in ADCC reactivity.

## DISCUSSION

As discussed in an earlier paper, there are several ways to test ADCC against EBV-specific antigens (Jondal, 1977). In the present paper we used a procedure which was found optimal for the EBV system, i.e. sera were mixed with supernatant material from the EBV-producing B-95-8 cell line and with freshly isolated allogeneic lymphocytes and Daudi target cells. Daudi cells do not express EBV surface antigens but have a high amount of Fc receptors, and evidence has been presented which indicate that EBV-specific immune-complexes are formed, during ADCC, which induce cytotoxicity by forming Fc–Fc receptor bridges between effector and target cells. From this follows that the reactive antibodies not necessarily are directed against MA but could be specific for other EBV-related antigens.

The apparent lack of correlations between ADCC and conventional anti-EBV titres, and between ADCC and clinical events, may therefore not be surprising. While earlier studies suggested a correlation between EBV neutralization and anti-MA reactivity of sera, later work has clearly demonstrated that neutralization could be achieved by low anti-MA reactive sera and this was speculated to depend on the

presence of additional antigenic specificities on the viral particles (de Schryver *et al.*, 1976). One of the patients in the present study, Salim, had in fact unchanged virus neutralizing activity in his sera during the anti-MA titre drop in the late recurrence period (Gunven *et al.*, 1973). This may well explain why even the low anti-MA titred sera were capable of forming immune complexes with EBV, inducing ADCC. Furthermore, there may be no reason to assume *a priori* a simple relationship between ADCC and tumour development as the formation of anti-MA antibodies most probably depends on differentiation towards virus production, a process which kills the host cell and thus is incompatible with tumour growth.

We have earlier shown that patients with acute infectious mononucleosis, a primary EBV infection, develop killer T cells directed against cell lines which carry the virus and that the specificity of these T cells is entirely distinct from serologically defined EBV antigens (Svedmyr & Jondal, 1975). Even in biopsy, material from BL cytotoxic T cells with a similar specificity have been found (Jondal *et al.*, 1975). It is thus possible that ADCC acts against EBV production whereas T-cell immunity is more important in the protection against tumour development.

This work was supported by the Swedish Cancer Society and by the Cancer Society of Stockholm and by Contract No. NO1 CP 33316 within the Virus Cancer Program of the National Cancer Institute.

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