Preliminary Seroepidemiological Studies on the Human Syncytial Virus

By B. G. ACHONG AND M. A. EPSTEIN

Department of Pathology, The Medical School, University of Bristol, University Walk, Bristol BS8 1TD, U.K.

(Accepted 10 February 1978)

SUMMARY

The seroepidemiology of the human syncytial virus was investigated by means of an indirect immunofluorescence test on 241 sera from Kenya, Tunisia, Singapore and Britain. These included sera from patients with nasopharyngeal carcinoma, other tumours of the oro-nasopharynx, tumours of other parts of the body, and from normal donors. In this study, the virus was found to infect only Kenyan Africans and all but one of these seropositive subjects had tumours, particularly of the oro-nasopharyngeal spaces. The significance of these findings is discussed.

INTRODUCTION

After the discovery of the human syncytial virus (Achong et al. 1971) several of its biological characteristics were defined (Epstein et al. 1974), but further investigations were hampered by the extreme cell-association of the agent and by the lack of an antiserum. Thus, it was clear that the virus belonged to a group including the simian (Clarke & Attridge, 1968; Clark et al. 1969), feline (Riggs et al. 1969; Hackett et al. 1970; McKissick & Lamont, 1970) and bovine (Malmquist et al. 1969; Boothe et al. 1970; Clarke & McFerran, 1970) syncytial agents by morphological criteria (Achong et al. 1971) and the possession of reverse transcriptase (Loh et al. 1977), and was immunologically distinct (Epstein et al. 1974). However seroepidemiological studies only became possible with the development of a tissue-culture adapted stain, an in vitro assay, and a specific immunofluorescence test (Loh et al. 1977).

The present paper reports on the incidence in various human populations of naturally occurring antibodies to the virus as determined by an indirect immunofluorescence test.

METHODS

Cells. Whole human embryo fibroblasts (HEF; Flow 5000), obtained from Flow Laboratories (Irvine, Scotland) were used between the 21st and 36th passages and were grown at 37 °C in Eagle's basal medium supplemented with 10% foetal calf serum, 1% non-essential amino acids, 0.08% bicarbonate, 100 units/ml penicillin and 100 μg/ml streptomycin.

The cells were grown in plastic tissue-culture flasks and were removed into suspension, when required, by treatment with 0.05% trypsin and 0.1% EDTA in phosphate-buffered saline (PBS).

Virus. Virus pools were prepared in 800 ml plastic tissue-culture flasks by removing the medium from subconfluent HEF and inoculating with 8 to 10 ml of undiluted cells and medium from infected cultures (Epstein et al. 1974). After 3 h of adsorption at 37 °C with
intermittent shaking, 70 ml of fresh medium were added. When a good cytopathic effect (Epstein et al. 1974) involving more than 80% of the cells was observed, the infected cultures, both cells and medium, were harvested and stored at -70 °C in small volumes.

**Preparation of infected HEF cells for immunofluorescent staining.** HEF cell suspension (1 to 2 ml) in medium, containing about 3.5 x 10^5 cells per ml, was seeded into 60 mm plastic Petri dishes containing 16 mm diam. coverslips, 6 ml medium were added and the Petri dishes incubated at 37 °C. When the cells had grown evenly over the coverslips the medium was removed and 1 ml of virus pool was added. After gently rocking to distribute the inoculum evenly, the virus was allowed to adsorb at 37 °C for 3 h and 6 ml fresh medium were introduced. About 72 h after inoculation, the coverslips were removed, washed with PBS, fixed in cold acetone for 10 min, and stored at -70 °C in sealed plastic bags.

**Immune sera.** Rabbit antisera to the human syncytial virus were prepared and used to make fluorescein-conjugated gamma-globulin fractions by methods already described (Loh et al. 1977).

**Indirect immunofluorescence.** Infected coverslip cultures, prepared as described above, were first exposed to serum diluted 1:4 in PBS for 1 h at 37 °C in a moist chamber. The coverslips were then transferred to a rack and washed for 15 min in a dish containing PBS, agitated by a magnetic stirrer, then washed by dipping ten times in each of two beakers of fresh PBS and then exposed to fluorescein isothiocyanate-conjugated anti-human immunoglobulin (Wellcome Reagents Limited, Wellcome Research Laboratories, Beckenham) diluted 1:20 in PBS. After incubation in a moist chamber for 1 h at 37 °C, they were washed in PBS as before, mounted, cells down, on glass slides in a PBS/glycerol (1:1) mixture and examined in a Leitz Wetzlar u.v. microscope. The following controls were always included. (1) Positive serum controls. Infected coverslip cultures stained by the direct immunofluorescence method with conjugated rabbit antisera (Loh et al. 1977) served as positive controls until positive human sera were found; thereafter, a positive human serum, stained by the indirect immunofluorescence method, was used. (2) Antigen controls. Infected coverslip cultures incubated with PBS instead of positive serum and then fluorescent antibody, and uninfected coverslip cultures incubated with positive serum and then fluorescent antibody, served as antigen controls.

**Human test sera.** Sera from four different regions of the world were obtained from patients with nasopharyngeal carcinoma (NPC), other malignancies of the oro-nasopharyngeal space, malignancies of other parts of the body, and from healthy adults. For such sera from Africa we are indebted to George Klein (Department of Tumor Biology, Karolinska Institutet, Stockholm, Sweden) and Peter Clifford (when in Africa, Department of Head and Neck Surgery, Kenyatta National Hospital, Nairobi, Kenya), from Tunisia and Singapore to Guy de Thé (Unit of Biological Carcinogenesis, International Agency for Research on Cancer, Lyon, France) and from Britain to Peter Clifford (Royal Marsden Hospital, Fulham Road, London). Other adult sera were furnished by the South West Regional Transfusion Centre, Southmead Road, Bristol, and by staff members of this Department.

**RESULTS**

In the first test, infected coverslip cultures stained by the direct immunofluorescence method with conjugated rabbit antisera gave a very bright cytoplasmic fluorescence (Fig. 1 a); these were used as positive controls, as mentioned above, until positive human sera were found; thereafter, a positive serum, stained by the indirect immunofluorescence method, was used.
In the indirect immunofluorescence tests, positive sera gave an extremely vivid cytoplasmic fluorescence (Fig. 2a) in the large multinucleated syncytial cells which generally showed the presence of 8 or more nuclei and extensive cytoplasmic vacuolation (Fig. 2a and b). Uninfected coverslip cultures incubated with positive serum and then fluorescent antibody failed
Fig. 2. (a) Infected HEF treated with human serum and then fluorescein-conjugated anti-human immunoglobulin. There is an extremely vivid cytoplasmic fluorescence in this indirect immunofluorescence test in the large multi-nucleated cells (cf. Fig. 1 b) indicating specific reactivity in the human serum tested. Magnification $\times 450$. (b) The same field as above seen by phase-contrast microscopy. Here the extensive cytoplasmic vacuolation and the numerous nuclei (8 or more) of the syncytial cells are clearly seen. Magnification $\times 450$. 
Table 1. Incidence of positive reactions in indirect immunofluorescence tests

<table>
<thead>
<tr>
<th>Source of sera (totals in parentheses)</th>
<th>Diagnosis</th>
<th>No. of individuals</th>
<th>Fluorescence</th>
<th>Per cent+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kenya (97)</td>
<td>NPC</td>
<td>42</td>
<td>+</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Other tumours of oro-nasopharynx</td>
<td>16</td>
<td>+</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Tumours of other parts</td>
<td>12</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>BL</td>
<td>8</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>19</td>
<td>+</td>
<td>18</td>
</tr>
<tr>
<td>Tunisia (50)</td>
<td>NPC</td>
<td>4</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Other tumours of oro-nasopharynx</td>
<td>13</td>
<td>+</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Tumours of other parts</td>
<td>10</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>23</td>
<td>+</td>
<td>23</td>
</tr>
<tr>
<td>Singapore (20)</td>
<td>NPC</td>
<td>10</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>10</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>Great Britain (74)</td>
<td>NPC</td>
<td>2</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Other tumours of oro-nasopharynx</td>
<td>18</td>
<td>+</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Tumours of other parts</td>
<td>1</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>53</td>
<td>+</td>
<td>53</td>
</tr>
</tbody>
</table>

Table 2. Titration of sera in indirect immunofluorescence tests

<table>
<thead>
<tr>
<th>No.</th>
<th>Diagnosis</th>
<th>Fluorescence with serum diluted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:4</td>
</tr>
<tr>
<td>NP 26</td>
<td>NPC</td>
<td>+</td>
</tr>
<tr>
<td>NP 35</td>
<td>Reticulum-cell sarcoma of post-nasal space</td>
<td>+</td>
</tr>
</tbody>
</table>

to elicit any fluorescence (Fig. 1 b); similar negative results were seen when infected coverslip cultures were incubated with PBS and then fluorescent antibody.

Sera from Kenya, Tunisia, Singapore and Britain (241 in all) were tested; these included sera from NPCs and normal donors from all four regions, and from patients with other tumours, both of the oro-nasopharynx and other parts of the body. The results are set out in Table 1.

Antibodies to the virus were not detected in 50 sera from Tunisia (4 NPCs, 13 other tumours of the oro-nasopharynx, 10 tumours of other parts of the body and 23 normal donors), 20 sera from Singapore (10 NPCs and 10 normal donors), and 74 sera from Britain (2 NPCs, 18 other tumours of the oro-nasopharynx, one other tumour and 53 normal donors).

In striking contrast, of the 97 sera from Kenya, 17 (17%) had antibodies to the virus. The incidence of positive sera in these 97 Kenyan specimens can be broken down thus: 10 (24%) positive out of 42 NPCs, 4 (25%) out of 16 other tumours of the oro-nasopharynx, 2 (17%) out of 12 tumours of other parts of the body, 0 (0%) out of 8 African (endemic) Burkitt's lymphomas (BL), and 1 (5%) out of 19 normal donors.

Two positive sera were titrated by the indirect immunofluorescence technique and the results (Table 2) showed that sera which were strongly positive at 1:4 dilution could be diluted as much as 32- or 64-fold and still give definite, if weak, fluorescence.
DISCUSSION

Although the human syncytial virus was originally isolated from cultures of a human tumour, it was thought important from the outset to be sure that the agent was indeed a virus of man, and not merely one of the related animal syncytial viruses which had gained access to the cultures. Certainly the original cultures were always maintained on human serum, thus precluding the inadvertent introduction of an animal virus in serum of animal origin (Achong et al. 1971), and investigations using specific antisera raised against the known animal members of the syncytial virus group showed that the human virus, while slightly related immunologically to simian foamy virus type 6 and the bovine syncytial virus, was a distinct entity (Epstein et al. 1974). Indeed, this double relatedness seems in itself to support the uniqueness of the human-derived virus, since an agent with such cross-reactivities has not been reported.

However, of far more importance in the context of the agent's infectivity for man is the incidence of specific antibodies in humans. The development of a monolayer culture system for the virus together with a specific rabbit-antiserum (Epstein et al. 1974; Loh et al. 1977) made surveys for such naturally occurring human antibodies possible, and the present findings now show unequivocally that the human syncytial virus indeed infects certain populations.

The virus does not appear to be a ubiquitous agent with a world-wide distribution since no antibodies to it were detected in 144 sera from donors in Britain, Tunisia and Singapore. On the other hand, Kenyan Africans have a substantial incidence (17%) of positive antibody reactivity, and it is of particular significance that most of these positive reactions were seen in patients with tumours, in contrast to only one out of 19 normal Kenyan donors.

With regard to the incidence of specific antibodies among tumour patients, those with NPC showed no greater evidence of infection (24%) than did patients with other tumours of the oro-nasopharynx (25%), and even these incidence rates were hardly different from the 2 out of 12 seropositives (17%) amongst patients with tumours of other parts of the body. As for the tumours which were accompanied by antibodies to the virus, no particular type predominated since this group included a variety of carcinomas, a melanoma, and 2 reticulum cell sarcomas. In view of the high incidence of antibodies to the virus amongst NPC patients and those with other oro-nasopharyngeal tumours (Table 1), it is surprising that no individual with antibodies was detected among the 8 patients with BL since many of these had lesions communicating with the mouth and nasal spaces.

Although the present findings do not indicate any special relationship of the human syncytial virus to NPC (from a case of which it was first isolated), they do confirm solidly that infections occur frequently (17%) amongst Kenyans and with an especially high incidence in those with tumours. The reason why the virus infects only Kenyans among subjects investigated from four different regions of the world is not clear and the nature of its association with tumours is obscure, but in view of the possession by syncytial viruses of a reverse transcriptase (Parks et al. 1971; Parks & Todaro, 1972; Loh et al. 1977) comparable to that of oncornaviruses (Gallo, 1972), this tumour association may well prove significant and certainly calls for further investigation.

The work was assisted by the Cancer Research Campaign, London, England, out of funds donated by the Bradbury Investment Co. of Hong Kong. The authors are most grateful to Mr J. Baker and Mr G. Ball for invaluable help.
Seroepidemiology of the human syncytial virus

REFERENCES


(Received 20 December 1977)