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THE PROPERTIES OF NON-VIRUS-PRODUCING (NP) ROUS SARCOMA CELLS*

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Abstract—NP tumours derived from pocks on the chorioallantoic membrane induced by high dilutions of RSV(B) and cultivated *in ovo*, probably contain transplantation-type antigens similar to those induced *in vivo* by high concentrations of RSV(B).

A significant number (6/21) of chickens in which NP tumours had regressed also had RSV(B)-neutralizing antibodies in their sera.

IT HAS been known for many years that some of the chicken tumours induced by very small amounts of the Bryan strain Rous sarcoma virus [RSV(B)] fail to yield infective virus following the usual extraction techniques. The reason for this was not apparent until Rubin and his colleagues discovered that RSV(B) was a defective virus and that another virus (RAV) was required to infect the transformed cell before the transforming virus could be synthesized. The tumour cells which were unable to synthesize RSV(B) before superinfection were termed "non-producer" (NP).

Some of the properties of NP cells, which had originated after *in vitro* transformation of chick embryo fibroblasts, were studied *in vivo* by Hanafusa, Hanafusa and Rubin[3]. In order to culture these cells *in vitro* they had to be mixed with $10^2 \times$ the number of normal chick embryo fibroblasts and it was such a mixture that was inoculated into chickens.

The American workers observed that (a) all the tumours produced regressed after about 12 days but that some re-appeared within another week and then regressed; (b) no virus-neutralizing antibodies appeared in the sera of the birds and (c) the birds were just as susceptible to subsequent infection with RSV(B) as normal birds of the same age. From these results they concluded that these NP cells contain no RSV(B)—specific coat antigens and no unique (transplantation) tumour antigens. NP cells, derived *in vitro* do, however, contain the avian leukosis group-specific complement-fixing ("Cofal") antigen (Dougherty and di Stefano[1]).

It seemed possible to us that the NP cells did not contain viral coat antigen but unlikely that, as transformed cells, they did not sensitize their hosts against a virus-induced transplantation antigen. Access to large quantities of NP cells enabled us to repeat these experiments.

* Professor Haddow first aroused my interest in avian tumour viruses in 1946. I have spent twenty years on one aspect or another of tumour virus research and it is not only an honour to be asked to contribute to this volume but fitting that this short paper be concerned with an aspect of avian tumour viruses.

RESULTS AND DISCUSSION

Experiments

When small quantities of RSV(B) (1–20 p.f.u.) are inoculated on to the chorio-allantoic membrane of the embryonated egg, pocks are produced. Each pock derives from infection with one transforming particle and may, or may not, produce virus on sub-cultivation, (Goldé [2]). Individual pocks were collected 7 days after virus inoculation of the eggs and further cultivated on the chorioallantoic membrane of eggs from our RIF-free Brown Leghorn (BL) flock. The pocks grow into small (*ca.* 3 mm dia.) tumours which may be subdivided and further cultivated in the same way. Virus-producing pocks are readily identified by the appearance of fresh pocks surrounding the tumour growth and these tumours were not further cultivated.

The details of four experiments with 67 individual pocks isolated from membranes having less than 20 pocks is shown in Table 1.

TABLE 1

Experiment	Pass No.	No. pocks or tumours	Died	V.P.	NP
A	1	9	4	1	4
	2	4	2	1	1
	3	1	—	—	1
	4	1	—	1	
B	1	10	5	2	3
	2	3	—	1	2
	3	2	—	1	1
	4–37–	1	—	—	1
C	1	34	25	7	2
D	1	14	8	1	5
	2	5	3	1	1
	3	1	—	—	1
	4	1	—	—	1
	5	1	—	—	1
	6	1	1	—	—

Thus, of the pocks which could be cultivated (25/27) eleven (44 per cent) were virus-producing (V.P.). A further five (20 per cent) yielded virus after sub-culture and only one (experiment B) remained NP for long enough to carry out transplantation experiments *in vivo*.

These cells, or those of primary chick-grown tumours derived from them, were grafted into the wing-web (at a dose of 10^5/bird) of more than 100 6-weeks old Brown Leghorn chickens of our RIF-free stock. Many (26 per cent) of the tumours which grew did not regress but enlarged progressively until the birds had to be killed. Others (20 per cent) grew and regressed. Some (33 per cent) grew, regressed and grew again, like those of Hanafusa *et al.*[3]

Transplantation resistance

Three groups of birds were further investigated for their resistance to transplantation-type antigen.

(1) Seven birds in which a tumour was actively growing were challenged in the other wing with a further graft of 10^5 NP cells to reinforce the immunological rejection which appeared to be responsible (in 20 per cent of the birds) for graft

regression. *None* of the *second* grafts grew and in five of the seven the *first* tumours also regressed.

(2) In this group the first tumours had all regressed by 28–35 days. The thirteen birds were then given 20 p.f.u. of the same virus (RSV(B)) into the opposite wing-web. *None* of the *first* tumours returned and *nine* of the *new* tumours which arose grew progressively. *Three* of the remaining four birds also developed tumours but these began to regress within a day or two of reaching maximum size and had completely disappeared within a week. The last bird's tumour also regressed under similar conditions but then began to grow again.

(3) In the third group of ten birds the *first* tumours had again regressed by 28–35 days. The group then received 20 p.f.u. of a different Rous virus strain (RSV (H)) in the opposite wing-web. Two of the *original* tumours grew within 7 days but both regressed 10 days later. The *second* tumours began to grow in all the birds, four grew progressively—including the two in the birds with *first* tumours. The remaining six became quite large (some 500–1000 mg estimated size) but all had regressed by the 70th day.

We conclude that NP cells cultivated *in ovo* do possess transplantation-type antigens but are only weakly immunogenic in the BL chicks and that resistance can be reinforced either by inoculating more NP cells (Experiment 1) or by producing new tumours with the same virus (Experiment 2). The new tumours, however, tended to grow progressively, presumably by overwhelming a weak immunity, or by infection of surrounding host cells.

Treatment with RSV(H), which is less virulent for BL chicks (Experiment 3), tended not to reinforce immunity against the NP cells and the RSV(H)-induced tumours themselves regressed in six out of ten birds.

Iso-antigenic differences between grafted NP cells and the host could be held to account for the results of Experiments 1 and 2 because the BL birds used are not inbred. However it is unusual in our experience for any tumours induced in young BL chicks with 20 p.f.u. of RSV(B) to regress and 3/13 disappeared completely in Experiment 2. This suggests that these three birds at least had been sensitized by RSV(B)-induced antigens in the NP cells.

Anti-viral antibodies

Sera of birds from these different groups were tested for capacity to neutralize RSV(B). A neutralization index (N.I.) greater than 0·5 was considered to be significant where N.I. = $\log_{10}$ no. of pocks in control group—$\log_{10}$ no. of pocks in test group.

Sera were heat inactivated before use and the final dilution was 1 : 10. Normal sera from untreated BL chicks of the same age was non-inhibiting.

Experiment 1: *Two* of the seven birds had anti-virus antibodies in their sera 19 days after inoculation of 10^5 NP cells. Following the second graft two further birds developed antibodies.

Experiment 2: In this group *three* out of seven birds tested had antibodies neutralizing RSV(B) at a time when the first tumours had regressed.

Experiment 3: In this group *one* out of seven sera tested at a time when the first tumours had regressed was neutralizing for RSV(B).

We conclude from these data that six NP tumours out of twenty-one tested were capable of synthesizing enough viral antigen to immunize their RIF-free hosts although

complete infective virus is not, apparently, synthesized in *detectable* quantities in the absence of super-infection by a RAV. This latter phenomenon would thus appear to be the only significant difference between NP cells and normal RSV(B)-induced avian tumour cells.

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