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AETIOLOGY OF JAAGSIEKTE: EXPERIMENTAL TRANSMISSION TO LAMBS BY MEANS OF CULTURED CELLS AND CELL HOMOGENATES*

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ABSTRACT

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Studies on the transmission of jaagsiekte (ovine pulmonary adenomatosis) both by subinoculation of cells of known sex and by cell homogenates into male and female lambs are reported. The results obtained indicate a thymocyte-dependent rejection of male cells in female recipients in contrast to the successful transplantation of male cells in male animals and female cells in both sexes. This suggests the presence of a surface antigen determined by the Y-chromosome in the tumour cells. A second mechanism of transmission, dependent on the transformation of the recipient's cells, was demonstrated by 2 cases of heterologous transplantation and confirmed by inoculation of cellular homogenates.

Résumé

ADENOMATOSE PULMONAIRE OVINE: TRANSMISSION EXPÉRIMENTALE AUX AGNEAUX AU MOYEN DE CELLULES EN CULTURE ET D'HOMOGENATS DE CELLULES

Des études sur la transmission de l'adenomatose pulmonaire ovine par la sub-inoculation de cellules de sexe connu et par des homogénats de cellules dans des agneaux mâles et femelles sont rapportées. Les résultats obtenus indiquent une réjection des cellules mâles par l'intermédiares de thymocytes dans les récipients femelles par contraste avec la transplantation fructueuse des cellules mâles dans les animaux mâles et des cellules femelles dans les deux sexes. Ceci suggère la présence d'une surface antigène déterminée par le Chromosome-Y dans les cellules de la tumeur. Un second mécanisme de transmission dépendant de la transformation des cellules du récipient a été démontré par 2 cas de transplantation hétérologue et confirmé par l'inoculation d'homogénats cellulaires.

INTRODUCTION

The transmission of jaagsiekte (ovine pulmonary adenomatosis) to new-born lambs by means of intratracheal inoculation of cells from an epithelial cell line derived from adenomatous lung tissue was reported by Coetzee, Els & Verwoerd (1976). The culture, which consisted of male cells, was established from the tumour of a female animal, suggesting that the known infectivity of the disease can be explained, at least in part, by the transmission of live tumour cells from animal to animal. To confirm and extend this observation, a number of additional cell lines were established from field cases of jaagsiekte, while 2nd generation cell lines were also derived from experimentally-produced cases. These various cell cultures, both male and female, as well as cell homogenates, were in turn inoculated into male and female new-born lambs and nude mice. The results of these studies and of attempts to increase the efficiency of transplantation by means of immunosuppression are reported in this paper.

MATERIALS AND METHODS

Cell cultures

Cell cultures were established from adenomatous lung lesions by the selective trypsinization procedure previously described by Coetzee *et al.* (1976). Enriched F12 medium (Weinstein, Orenstein, Gebert, Kaighn & Stadler, 1975), in which penicillin and streptomycin were replaced by ampicillin(3) at a concentration of 200 mg/ℓ, was used throughout. All cell cultures were routinely screened for possible mycoplasm infection by staining with bisbenzamide fluorochrome stain(1) (Chen, 1977). Cultures that stained positively were also tested for mycoplasm contamination by standard cultural procedures and discarded, if positive.

Karyotype analysis

Karyotype analyses were done on monolayers as described previously (Coetzee *et al.*, 1976). In some cases better results were obtained with the "drop" procedure described by Robinson, Bey, Alexander & Gear (1976).

Experimental animals

Dorper cross-bred sheep were used for most of the transplantation studies. A few experimental lambs and most of the field cases were pure-bred Merinos. Pregnant ewes were obtained from various sources and housed in experimental groups in open pens separated from other experimental animals.

Nude mice were either of an inbred Balb/c strain kept in a Trexler-type plastic isolator, as previously described (Verwoerd, Meyer-Scharrer & Du Plessis, 1977), or of an outbred strain derived from the HaICR mice and housed under "clean" laboratory conditions.

Tumorigenicity tests

Cells to be tested for tumorigenicity were harvested from monolayer cultures in glass flasks by trypsinization and suspended in growth medium at a concentration of 1 to 2×10^7/mℓ. New-born lambs (up to 1 week old) were injected intratracheally with 4–5 mℓ of this suspension (5×10^7 to 1×10^8 cells/lamb). In a few cases the same number of cells was injected both intratracheally and subcutaneously. Nude mice of weanling age were injected subcutaneously with 5×10^6 to 1×10^7 cells and kept under observation for 3 months. Autopsies were carried out on lambs

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either when clinical symptoms developed or after 12 months, if no symptoms were seen. The observation period was limited to one year to minimize the possibility of confusing experimentally-produced jaagsiekte with natural infection, as the latter is rarely seen before the age of 18 months to 2 years.

Preparation of cell homogenates

About 5×10^7 cells were harvested from monolayer cultures and suspended in 10 mℓ of 0,02 M tris buffer, pH 7,4. The suspension was then frozen and thawed 3 times and treated in a MSE ultrasonifier for 5 min at full power. One fifth of the suspension was diluted into growth medium in a plastic tissue culture flask. A small aliquot was stained with a vital stain (1% nigrosine) and examined microscopically for the presence of live cells, while the rest was injected intratracheally into newborn lambs. The absence of cellular outgrowths in the culture flask after 14 days was taken as proof that the homogenate did not contain viable cells.

Histopathology

Specimens for histopathological examination were taken from the lungs of the majority of sheep, including all those which showed macroscopically visible lesions. These were processed by routine procedures, sections being stained with haematoxylin and eosin (HE). Jaagsiekte (pulmonary adenomatosis) was confirmed histologically in every case in which this diagnosis was made.

Immunosuppression

The preparation, assay and use of anti-thymocyte serum (ATS) and anti-macrophage serum (AMS) for the suppression of cellular immunity in new-born lambs have been described by Broekman, Eksteen & Verwoerd (1977). Both sera were administered intraperitoneally in 10 mℓ doses either alone or in combination, according to the schedules indicated in Table 4. In addition, 3 lambs were injected intratracheally with a suspension containing 100 mg of silica dust(1) 2 days before the cells were injected, in an attempt to destroy the alveolar macrophages (Zisman, Hirsch & Allison, 1970). Two lambs received similar doses of silica in combination with ATS.

Results

Tumorigenic epithelial cell lines

The characteristics of 11 epithelial cell lines which were established from jaagsiekte lesions are summarized in Table 1. Two male and 2 female cell lines were obtained from natural cases. Only line 15.4 is of a sex different from its donor animal. Five out of the 6 experimental cases of jaagsiekte from which cell lines could be established were produced by the inoculation of male 15.4 cells. Male tumours were obtained in male recipients, but a single female recipient gave rise to a female tumour cell line (58.2) (Fig. 1). The reciprocal injection of female 21.3 cells into a male recipient yielded a male cell line (71.1) (Fig. 1).

Of the 8 cell lines tested for tumorigenicity in lambs 2 gave negative results, but in both cases only 2 lambs were available for the test. That this number is too small for the result to be significant is indicated by the fact that one of these cell lines (48.2) was tumorigenic in nude mice. Only 3 out of 9 cell lines inoculated into nude mice proved to be tumorigenic in this host.

TABLE 1 Epithelial cell lines established from adenomatous ovine lung tissue

Cell line (sex)	Sex of donor	Cells inoculated	Tumours produced in	
			lambs	nude mice
9.11 (male)....	male	natural case	NT	NT
15.4 (male)....	female	natural case	18/29	14/17
21.3 (female)..	female	natural case	6/10	0/10
30.2 (female)..	female	natural case	0/2	0/10
29.3 (male)....	male	15.4 (male)	1/2	NT
45.3 (male)....	male	15.4 (male)	1/2	0/2
48.3 (male)....	male	15.4 (male)	0/2	1/10
58.2 (female)..	female	15.4 (male)	2/6	0/9
59.1 (male)....	male	15.4 (male)	1/1	6/10
71.1 (male)....	male	21.3 (female)	NT	0/6
69.2 (female)..	female	15.4 (male) cell homogenate	NT	0/10

Transformation as a possible mechanism of jaagsiekte transmission

Evidence that jaagsiekte can also be transmitted by an agent that transforms the recipients' cells was obtained in 2 ways. Firstly the transplantation of male 15.4 cells into a female lamb and of female 21.3 cells into a male lamb gave rise to tumours from which cell lines (58.2 and 71.1) were established, and these clearly possessed female and male karyotypes respectively (Fig. 1). All the tumours were examined histopathologically and showed the adenomatous features typical of jaagsiekte. Secondly, cell homogenates free from viable cells were prepared from male 15.4 cells (see Materials and Methods) and injected intratracheally into 8 new-born lambs. Four of these lambs developed typical jaagsiekte lesions (Table 2, Fig. 2). A culture (69.2) established from the only positive female was found to be female (Fig. 1), proving that transplantation by means of residual male cells did not occur.

TABLE 2 The efficiency of transmission of jaagsiekte to new-born lambs by means of intratracheal injections of tumour cells and cell homogenates as a function of the sex of both the cell line and the recipient

Cell lines	Male recipients		Female recipients	
	Transmissions	Efficiency	Transmissions	Efficiency
Male: 15.4, 29.3, 45.3, 59.1, 48.3.....	13/17	76%	8/19	42%
Female: 21.3, 58.2, 30.2	3/8	37%	5/10	50%
Cell homogenate (15.4)	3/5	60%	1/3	33%

(1) Dowson & Dobson, Johannesburg (Superfine, particle size range 0,8–2,0 μm)

Sex-dependent susceptibility of lambs to transplantation of jaagsiekte cells

The tumorigenicity data given in Table 1 suggest an average efficiency of transplantation in lambs of about 50% for both male and female cells. When the figures in Table 1 are analysed according to the sex of the recipient lambs, however, the transplantation efficiency of male cells seems to be significantly higher in the homologous than in the heterologous sex (Table 2). The effect is even more pronounced when the growth rate of the tumours is taken into account (Table 3). In male recipients transplantation of male cells produced fast-growing tumours with extensive lesions often involving $\frac{1}{3}$ of the lungs or more after 6–12 months (Fig. 3). In contrast with this, the same cells produced mainly early lesions (Fig. 4) or small nodules (Fig. 5) in female recipients after 12 months.

In the case of female cells the number of experimental animals involved was smaller and the results consequently more difficult to interpret. There does not seem to be a significant difference, however, between either the efficiencies of transplantation (Table 2) or the growth rates of the tumours (Table 3) between recipients of the 2 sexes.

Transmission by means of homogenates of male cells followed a distribution pattern similar to that of intact male cells.

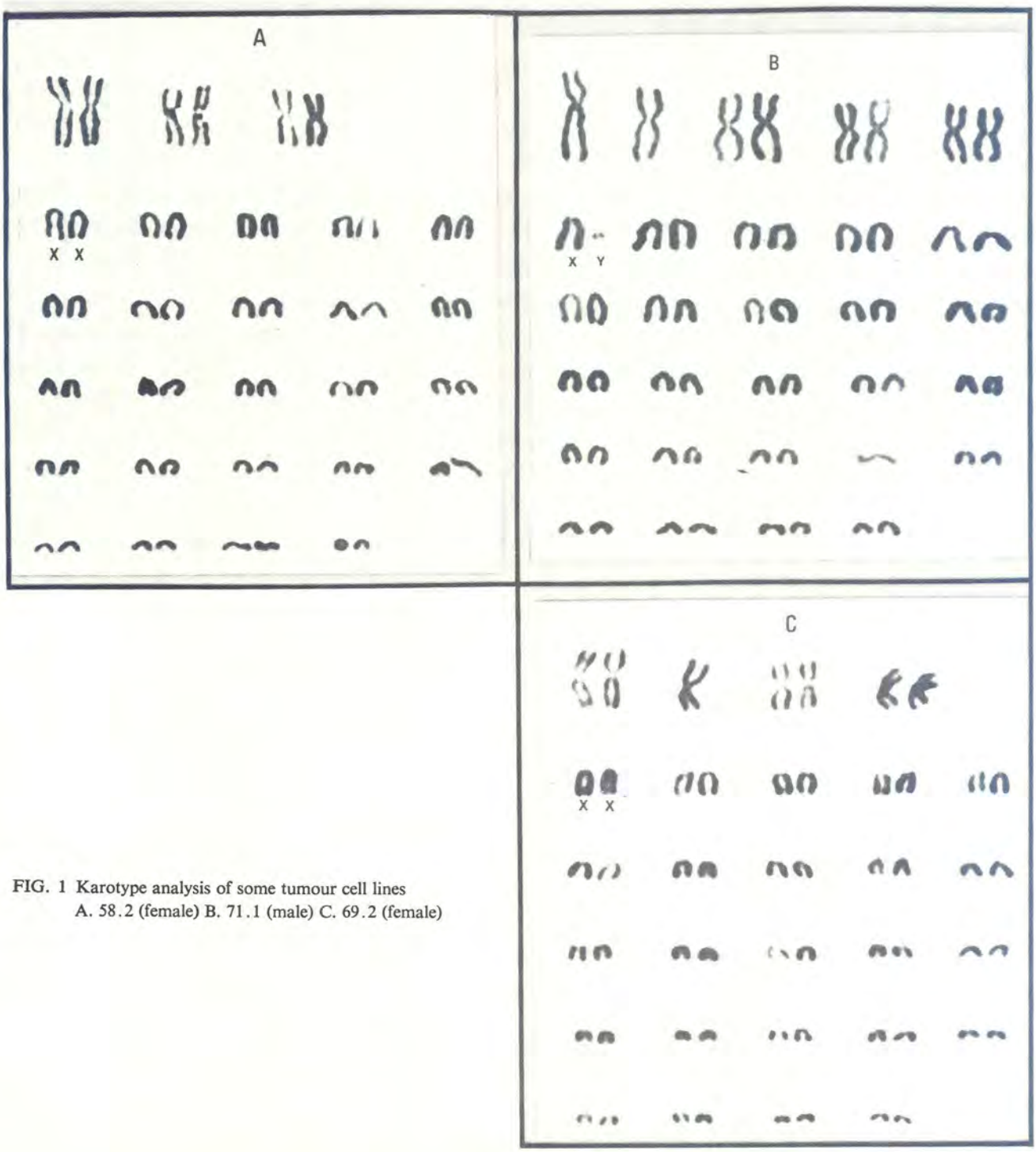
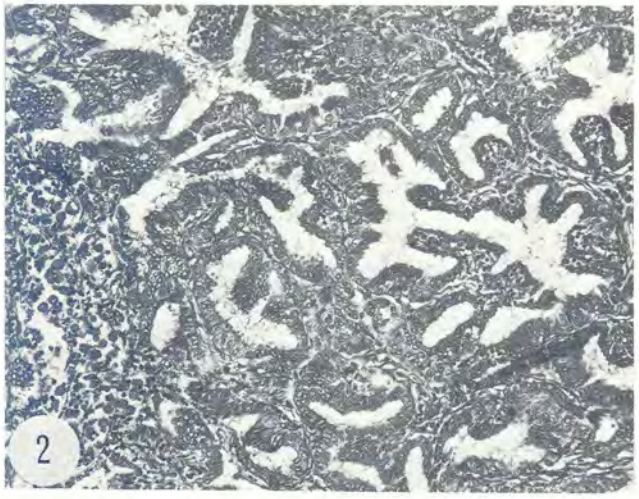
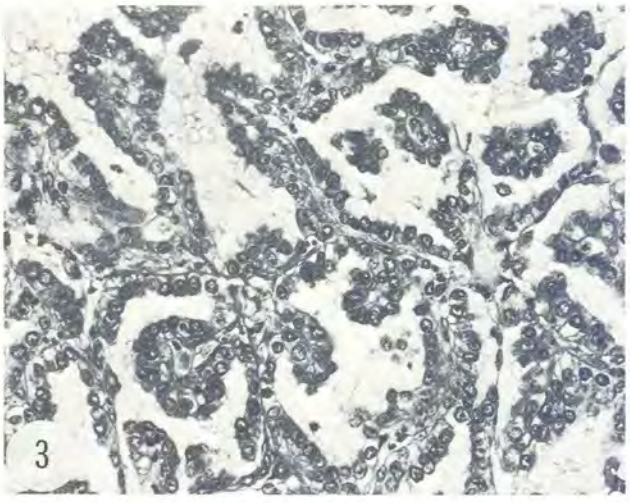
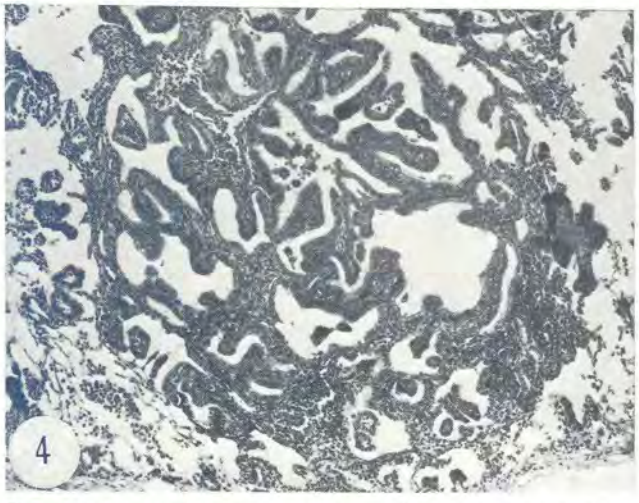


FIG. 1 Karotype analysis of some tumour cell lines
A. 58.2 (female) B. 71.1 (male) C. 69.2 (female)







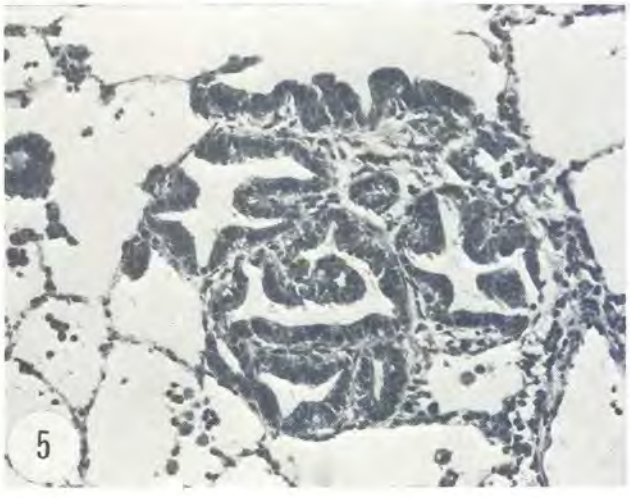


FIG. 2–5 Histopathology of experimentally produced jaagsiekte lesions

FIG. 2 Extensive lesions produced by the intratracheal inoculation of a homogenate of male cells into a female recipient. A female tumour resulted (karotype in Fig. 1 C), confirming transformation of the recipient's cells. HE×120

FIG. 3 Extensive lesions produced by the transplantation of male cells into a male recipient. HE×300

FIG. 4 Early lesion produced by the inoculation of male cells into a female recipient (male tumour, transplantation). HE×75

FIG. 5 Small focal lesion produced by the inoculation of male cells into a female recipient (female tumour, transformation). This tumour gave rise to cell line 58.2, the karyotype of which is shown in Fig. 1 B. HE×120

TABLE 3 Growth rates of tumours produced in male and female lambs by intratracheal injection of male and female cells

Growth rate of tumour	Male cells		Female cells	
	Male lambs	Female lambs	Male lambs	Female lambs
4+: Extensive lesions within 6 months	2	0	2	0
3+: Extensive lesions after 12 months...	9	2	0	2
2+: Small lesions after 12 months...	2	3	1	2
1+: Early lesions after 12 months...	0	4	0	1

Effect of immunosuppression on the transplantation of jaagsiekte cells

In the experiments summarized in Table 4, an attempt was made to improve the efficiency of transplantation of 15,4 cells by suppressing the cellular immune responses usually responsible for the rejection of tumour transplants. Anti-thymocyte serum (ATS) and anti-macrophage serum (AMS) were used in various combinations as shown. The efficiency of these reagents in lambs have previously

TABLE 4 Influence of immunosuppression on transmission of jaagsiekte to new-born lambs by transplantation of 15.4 cells

Immunosuppression	Males		Females	
	Trans-mission	Average growth rate*	Trans-mission	Average growth rate*
None.....	5/5	+++	3/9	+
ATS (D-1, +1, +3, +5, +7).....	1/3	+++	1/2	++
AMS (D-1, +1, +2, +5, +7).....	0/2	—	0/2	—
ATS+AMS (D-1, +1, +3, +5, +7).....	0/3	—	0/2	—
ATS (D-3 to D+21)...	2/2	++++	2/3	++
ATS+AMS (D-3 to D+21).....	2/2	++++	1/2	++
ATS+silica.....	2/2	++	NT	—
Silica.....	2/2	+++	0/1	—

* Growth rates scored as in Table 3

been demonstrated (Broekman, *et al.*, 1977). In addition, silica particles of defined size distribution were administered intratracheally in an attempt to inactivate alveolar macrophages (Zisman *et al.*, 1970).

All of the male lambs not receiving immunosuppressive treatment developed extensive lesions, whereas the small number of equivalent females that were positive had small lesions. The trend shown in Table 3 is thus confirmed. Treatment with ATS obviously could not further facilitate the transplantation in male recipients, but in the case of females it did seem to enhance both the number of positives and the growth rate. Treatment with AMS alone or in combination with a short course of ATS completely blocked transplantation in both sexes. Silica dust did not have any significant effect as far as can be judged from the small number of animals involved.

Discussion

The establishment of the 15.4 cell line from adenomatous ovine lung tissue has previously been reported (Coetzee *et al.*, 1976). It was shown that the cells of this line are male in character, even though derived from a female animal, and this suggests that jaagsiekte is transmitted in nature by means of the transplantation of viable cells via an aerosol or in some other way. Since that report we have established 10 more cell lines, determined their karyotypes and tested them for tumorigenicity in both lambs and nude mice. In 8 out of 10 cases the sex of the cell line was identical with that of the donor in field cases and of both donor and injected cells in experimental cases. This is consistent with transplantation as the mechanism of transmission, though transformation cannot be excluded as mechanism.

The female 58.2 cell line, however, originated from the transplantation of male cells into a female animal and the male 71.1 line from inoculation of female cells into a male animal. The only possible explanation for this phenomenon is that the recipient's cells are transformed by the transfer of genetic information, viral or otherwise, present in the transplanted cells. Transformation as an alternative mechanism for the oncogenesis of jaagsiekte was confirmed by the transmission of the disease with cell homogenates. Unequivocal proof that these results were not due to the presence of any residual viable cells was provided by the cell line 69.2, derived from a female animal injected with homogenates of male cells, which was clearly female in its karyotype. All the tumour cell lines were aneuploid, without any characteristic deviation in chromosome number or morphology.

During the course of experiments aimed at the improvement of the transplantation efficiency by means of immunosuppression, it became obvious that the sex of the recipient lambs influenced both the percentage of animals in which tumours developed and the growth rates of the resultant tumours, especially when male cells were injected. An analysis of all the experimental transplantation attempts carried out with the various cell lines, as summarized in Tables 1 and 2, confirmed this impression in the case of male cells, whereas transplantation of female cells was equally effective in both sexes. It should be noted that animals which received immunosuppressive treatment with AMS are not included in Table 2 because of its blocking effect in both sexes. Those treated with ATS are included in the 36 that were injected with male cells, because it was shown that this treatment has no effect on the transplantation efficiency in male recipients, and only a mild enhancing effect on transplantation in female recipients. When the latter effect is taken into account, the transplantation efficiency of male cells in female recipients is probably lower than the 42% indicated in Table 2, which would make the difference between the 2 sexes even more pronounced.

The enhancing effect of ATS suggests that the lower efficiency of transplantation of male cells in females is due to an immunological rejection mechanism. The observed phenomenon could in fact be explained rather well by the presence of a surface antigen determined by the Y-chromosome in male cells. Such cells would be recognized as foreign and rejected in female animals, but not in males. Female cells, on the other hand, would not possess this antigen and should be accepted equally well by both sexes. Such an antigen, termed the H-Y antigen, has in fact been demonstrated in mice and various other animals (Silvers & Wachtel, 1977). In most cases, however, it is a rather "weak" antigen playing a minor role in transplantation compared to the dominant histo-compatibility antigens. Little is known about either the H–Y antigen or the transplantation antigens of sheep, therefore their relative importance in the transplantation studies described cannot be evaluated.

It should furthermore be emphasized that the injection of tumour cells into the lungs, where they presumably attach to and proliferate on the surface of the alveolar epithelium, cannot be regarded as a normal transplantation process subjected to the usual rejection reactions. This is borne out by the relatively high percentage of tumour development found in animals that were completely random-bred and also genetically unrelated to the injected cells. The relative absence of normal immunological rejection reactions in the lung alveoli is also illustrated by the fact that a number of lambs that were injected at the same time intratracheally and subcutaneously with 15.4 cells developed jaagsiekte lesions in the lung, but subcutaneous tumours were never seen (unpublished observations).

The transmission of jaagsiekte by means of cell homogenates would not be expected to be influenced by the sex of the cells from which it was derived or of the recipient if an H–Y antigen is responsible for the sex-dependent transplantation effect. The data given in Table 2 unexpectedly do seem to suggest a similar dependence of transformation on sex. The numbers involved are small, however, and the effect would have to be confirmed by further studies before an attempt is made to explain it.

With the exception of ATS treatment in female lambs receiving male cells, attempts to increase the transplantation efficiency by immunosuppression failed. Treatment with AMS alone or in combination with a short course of ATS seemed to block the transplantation of 15.4 cells in both male and female recipients completely. More extensive treatment with ATS apparently neutralized this effect. These results suggest a role for the thymocytes in a sex-dependent rejection of tumour cells and an enhancing role for the alveolar macrophages in susceptible recipients.

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