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Plumage Colour Gene (i^+), a Possible Modifier on Cellular Susceptibility to RSV (RAV 49) in White Leghorn Fowl

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Summary. Seventy-six embryos from three isozygous Reaseheath lines I, C and W and 336 embryos derived from W × R line inbred parents, of White Leghorn fowl, segregating for plumage colour genes and tumour virus genes controlling susceptibility to an avian tumour virus of subgroup C, RSV(RAV49), were studied to examine whether the epistatic dominant white (*I* gene) and its recessive allele (i^+ gene) in the host genome modified susceptibility to RNA tumour viruses.

It was observed that the recessive allele of the dominant white gene modified the incidence of tumour pocks on the chorioallantoic membranes of embryos inoculated with RSV(RAV49). In the segregating W × R population, susceptible black embryos had on average 46 pocks fewer than the white susceptible embryos which had about 119 pocks.

It was further shown in this stock of birds that the tumour virus genes and the plumage colour genes for dominant white are located on different chromosomes.

Introduction

Embryos of the highly inbred ($F > 0.99$) Reaseheath isozygous lines I, C, W and R of White Leghorn fowl showed varying degrees of response of the chorioallantoic membrane (CAM) to subgroup C avian tumour viruses, MH_2 reticuloendothelioma virus and RSV(RAV49) (Payne and Biggs, 1970). The susceptibility of these four lines to subgroup C virus as measured by the average pock count on the CAMs covered a descending order of magnitude. Genetic tests involving the cross between the last two lines (W and R) of the series elucidated the mode of inheritance of resistance to this virus subgroup to agree with a simple Mendelian monohybrid theory (Payne and Biggs, 1970). Hence the W and R lines were assigned $c^s\,c^s$ and $c^r\,c^r$ genotypes respectively in accordance with the nomenclature proposed by Crittenden *et al.* (1967).

Because the relative susceptibility of the I and C lines was more than the W line, these lines therefore can be also assigned $c^s\,c^s$ genotype on a two alleles system at the tumour virus C (*tvc*) locus similar to the *tva* and *tvb* loci.

The least susceptibility of the W line may be due to one or more of the following reasons influencing either individually or in combinations.

1. Differential expressivity of the $c^s\,c^s$ genotype in different inbred strains may be operating due to unknown reasons.

2. Polyallelism may be in existence at the *tvc* locus, hence different alleles coding for different grades of susceptibility may have been randomly fixed in these inbred lines.

3. Influence of other major host genes to modify the phenotypic expression of the $c^s\,c^s$ genotype may be another possibility.

4. Many minor modifier host genes may be influencing the phenotypic expression of the tumour virus genotype and therefore the response to subgroup C avian tumour viruses may be of a polygenic trait in disguise of a monogenic inheritance.

Crittenden et al. (1969) observed differential response patterns to infection by subgroup C virus for the heavy and light breeds of fowls. An inhibitor gene (I^e) discovered by Payne *et al.* (1971), which has been shown to influence and modify the expression of the susceptibility gene (e^s) for the response to infection by subgroup E virus, RSV(RAV-0). In mice the incidence of a reticular neoplasm has been reported to be associated with A^y yellow coat gene (Deringer, 1970).

Therefore it was of interest to investigate the cause of least susceptibility of the W line as compared to the other two inbred lines. I, C and R lines are homozygous for the epistatic dominant white gene (*I*) and the W line is homozygous for its recessive allele (i^+) using the gene designation of Smith and Nordskog (1963). The development of plumage colour difference in these lines was described by Pease (1948).

Under a complete dominance hypothesis at each of the two loci controlling the plumage colour and susceptibility to virus infection and due to random re-association of the genotypes four possible phenotypic variants, white susceptible ($I\text{–}c^s\text{–}$), white resistant ($I\text{–}c^r\,c^r$), black susceptible ($i^+\,i^+\,c^s\text{–}$) and black resistant ($i^+\,i^+\,c^r\,c^r$) can be recovered from a cross between $i^+\,i^+\,c^s\,c^s$ W line and $II\,c^r\,c^r$ R line. Hence on a quantitative basis the c^s-phenotypic expression for tumour pock incidence on CAM associated with I– and $i^+\,i^+$ genotypes can be studied to account for a linear or non-linear combination be-

tween these genotypes. Any non-linear effect therefore can be partially attributed to one of the many possible other causes of differential phenotypic expression of the c^s c^s genotype as envisaged earlier.

Thus studies involving the pure lines I, C, W and cross between the W×R lines are described to support the evidence of the modifying effect of the plumage colour gene on the sensitivity of the individuals of the tumour virus genotypes at *tvc* locus. The R line was not available for studies.

Material and Methods

Chick embryos:

11-day-old chick embryos from parents of the I, C and W lines and the randomly mated fifth generation of the W × R cross were obtained. For production of each type of embryo about 8 dams were mated to a single sire.

Strain of virus:

Subgroup C avian tumour virus ,RSV(RAV49) (Payne and Biggs, 1970) was inoculated in 0.1 ml volume at various dilutions depending on the type of embryos. Phosphate buffered saline (PBS) with 5% calf serum was used as a diluent. The procedure for inoculating 11-day-old chick embryos and counting pocks on the CAMs was that described by Dougherty *et al.* (1960). The titre of the virus stock on susceptible Brown Leghorn chick embryos was $10^{5.85}$ pock forming unit (pfu) per millilitre (ml).

Experimental design:

Experiment 1.

Inbred embryos were inoculated with virus diluted to give an average pock count of 70 or more. Eight I line, 55 C line and 13 W line embryos were inoculated.

Experiment 2.

Since the W line embryos were four times less susceptible than the I and C lines (Expt. 1) the segregating embryos from W and R cross were inoculated with four times more virus to facilitate the identification of resistant and susceptible embryos, as well as to make the c^s gene expression comparable to that of the c^s gene of the I and C lines. One hundred and sixty two embryos in five lots were challenged with a $10^{-2.4}$ dilution of virus. Pock counts on the CAMs and the down colour of the individual embryos were recorded. To avoid any bias in counting pocks on CAMs, counts were made by a technician who had no prior knowledge of any association between pock counts and embryo down colour.

Experiment 3.

In experiment 2 it was observed that the black susceptible embryos had a significantly lower average pock count on the CAMs than had the white embryos. Experiment 3 was conducted to test the hypothesis that black embryos were still relatively more resistant than white embryos when the virus dose was increased. The virus concentration was increased ten-fold above the concentration used in experiment 2. One hundred and seven embryos were challenged with virus in 4 lots.

Statistical and Genetical Analyses.

Classification of resistant and susceptible CAMs.

The pock counts of individual CAMs were transformed to Log_{10} (individual count + 1) to normalise the distribution of pock counts (Bower *et al.*, 1964; Payne *et al.*, 1971). The transformed counts were plotted on normal graph paper. From visual inspection of the distribution of the pock classes, a line was drawn between the two distribution modes to delineate the resistant and susceptible CAMs. At a virus dilution of $10^{-2.4}$, (Expt. 2), CAMs which had 5 or fewer pocks were considered resistant and those having more pocks were considered susceptible (Fig. 1). In experiment 3 where the virus concentration was ten-fold more than the concentration used in experiment 2, resistant embryos were expected to have a maximum of 50 pocks with very high pock counts ranging from 51 pocks to TNC (too numerous to count) for the susceptible ones (Fig. 2).

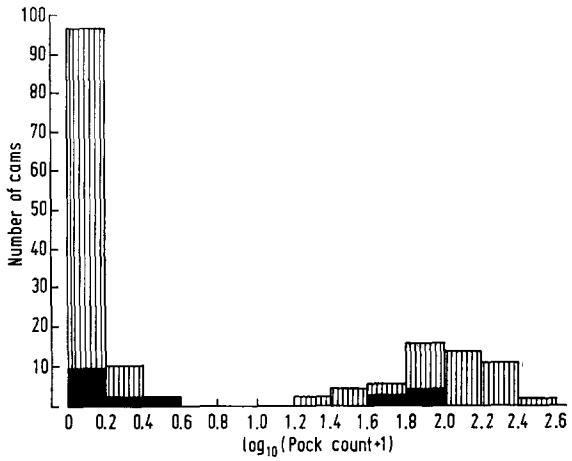

Fig. 1. Distribution of Log_{10} pock counts for W×R embryos inoculated with RSV(RAV49) at $10^{-2.4}$ virus dilution. The frequency of black embryos within pock count classes are shaded (Experiment 2)

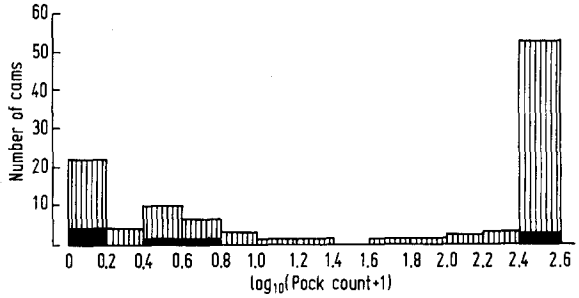

Fig. 2. Distribution of Log_{10} pock counts for W×R embryos inoculated with RSV(RAV49) at $10^{-1.4}$ virus dilution. The frequency of black embryos within pock count classes are shaded (Experiment 3)

Estimation of gene frequency for c^r and i^+:

In order to perform a genetic analysis of the data of experiment 2, it was necessary to estimate the gene frequencies in this population very precisely. The four phenotypes under the assumption of equilibrium of the gene and zygotic frequencies at the two loci are expected as follows:

Genotype	Phenotype	Expected frequency	Observed numbers
$I-c^s-$	White susceptible	$pq\,(2-p)\,(2-q)$	n_1
$I-c^r\,c^r$	White resistant	$(1-q)^2\,p(2-p)$	n_2
$i^+i^+c^s-$	Black susceptible	$(1-p)^2\,q(2-q)$	n_3
$i^+i^+c^r\,c^r$	Black resistant	$(1-p)^2\,(1-q)^2$	n_4

Where $(1 - p)$ and $(1 - q)$ are the respective gene frequencies of i^+ and c^r and n is the observed number in each subclass such that $\Sigma n = N$. The gene frequency of i^+ and c^r can be very precisely estimated by the use of the maximum likelihood estimating procedure (Mather, 1949 and Kempthorne, 1957). In terms of n, the $(1 - p)$ and $(1 - q)$ parameters are:

$$(1 - p) = \sqrt{\frac{n_3 + n_4}{N}} \quad \text{and} \quad (1 - q) = \sqrt{\frac{n_2 + n_4}{N}}.$$

Genetic model for analysis of the data of experiment 2:

A genetic model for estimation of the interaction deviation due to I and *tvc* loci were used according to the outline described by Falconer (1960). The model was:

$$\hat{G} = M + G_I + G_{tvc} + (I \times tvc)$$

Where $\hat{G}$ = estimated genotypic value

M = overall genotypic value for population under study

G_I = genotypic value of any individual attributable to plumage colour locus

G_{tvc} = genotypic value of an individual attributable to virus-induced tumour response locus

$I \times tvc$ = deviation due to the additive combination of the two genotypic values

If the interation deviation is not zero for any combination of genes at different loci, then a non-linear combination of the effects of the genes at the two loci under consideration is indicated and therefore the genotypic value would not combine additively.

Since in the present experiment the subclass phenotypes were distinguishable genotypically, the means for all phenotypic subclasses were identical to the means of the respective genotypes. The above model is therefore an appropriate one for estimating interaction between the two loci under consideration. Statistical analyses for Experiments 1, 2 and 3.

Experiment 1:

Since the embryos of the I, C and W inbred lines were assigned the genotype $c^s c^s$, under the assumption of a normal distribution of pock counts in the population, the difference between the mean pock count for each line would estimate the underlying line differences caused by other genes. However, by partitioning the two degrees of freedom for lines into appropriate single degrees orthogonal contrasts provide partial answers to the two following questions:

1. Are the mean pock counts on the CAMs of white embryos different from that of black embryos?
2. Do white embryos of the I and C inbred lines respond equally to the inoculated virus as measured by the mean pock counts?

Experiment 2:

Based on the gene frequency estimates the expected frequency of sub-classes were computed and then were compared with the observed numbers by the χ^2-technique to test the hypothesis that there was random association in this population of the genes of the tumour virus locus, *tvc* and the genes of the plumage colour locus. The subclass means in the log scale were analysed by the analysis of variance technique (Ostle, 1963) and three independent questions for which meaningful answers were sought were asked in relation to the proposed genetic model.

Experiment 2 and Experiment 3: (Combined analysis)

In experiment 3 ten-fold more virus was used than in experiment 2 and was conducted at a different time based on the results of experiment 2. Under a complete dominance at the two loci under study, four phenotypic variants as described earlier were expected regardless of dose of virus used. Also the proportion of resistant and susceptible subclasses within down colour phenotypes were expected to remain constant irrespective of the dose of virus input per embryo. Because experiments 2 and 3 with two doses of virus were performed at different times, the difference in observed frequencies of resistant and susceptible CAMs within dose was confounded with that of virus dose used at different times. Hence a danger of misrepresentation of the observed subclass phenotypes in different frequencies due to different times of virus inoculation leading to invalidation of the conclusions of the combined analysis of experiments 2 and 3 was required to be eliminated. Therefore, 67 eggs were divided into two groups and were challenged at the same time with the doses of virus used in experiments 2 and 3 respectively. The observed proportions of resistant and susceptible CAMs within dose were then compared with those of the experiments where doses of virus were given at different times. The validity of a combined analysis of experiments 2 and 3 was based on the non significant χ^2 value (1.08, $P > 0.05$). Data of experiments 2 and 3 were then combined for an analysis by the special χ^2 technique described by Kimball (1954) to test the hypothesis of whether or not the subclass phenotypic frequencies were contingent on the virus input per embryo. Here the contingency table for the χ^2 analysis was of 2×4 type, the two virus dilutions being the rows and the four subclass phenotypes the columns of the table. The three degrees of freedom available for testing the hypothesis were further partitioned into single degrees of freedom to provide an answer to the question: Do the white embryos become more sensitive than the black embryos when inoculated with a higher dose of virus, resulting in a significantly increased frequency of susceptible embryos?

Results

The frequency distribution of pock count classes in the log scale for all embryos of the three inbred lines, I, C and W is shown in Fig. 3. The average pock counts in the original scale for the three lines were 105.12, 81.04 and 21.08 respectively. Hence the relative sensitivities of the C and W lines as compared to the I line were 0.77 and 0.25 respectively. The estimate of the W line agreed well with that of Payne and Biggs (1970) who estimated it as 0.23 for the response to the same virus, RSV(RAV49).

The mean pock counts with standard errors and 99% confidence interval (C.I.) in the log scale for the

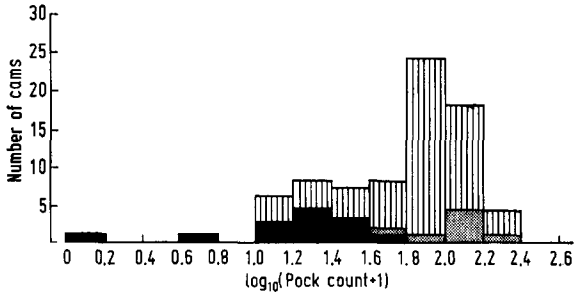

Fig. 3. Distribution of Log_{10} pock counts for all embryos of the inbred lines, which were inoculated with RSV(RAV49) at $10^{-3.0}$ virus dilution. The frequency of black embryos of the W line, the white embryos of the I line within pock count classes are shaded and cross hatched respectively (Experiment 1)

Table 1. *Means standard errors (S. E.)* and *99% confidence intervals (C. I.) for the three inbred lines, I, C, and W in the log scale of pock counts on the embryonic CAMs inoculated with RSV(RAV49) (Experiment 1)*

Line	No. of CAMs	Mean ± S.E.	Range of 99% C.I. on line mean
I	8	1.9850±0.1134	1.6845−2.2855
C	55	1.8334±0.0433	1.7186−1.9482
W	13	1.1992±0.0890	0.9633−1.4151

Table 2. *Analysis of variance for the mean pock counts (log scale) of three inbred line embryos, I, C, and W inoculated with RSV(RAV49) (Experiment 1)*

Sources	df	SS	MS	F-value
Inbred line	2	4.7621	2.3810	23.14**
Contrast 1 (White embryos vs Black embryo)	1	4.6017	4.6017	44.72**
Contrast 2 (I line White embryo vs C line White embryo)	1	0.1604	0.1604	1.56
Error	73	7.5141	0.1029	

** $P < 0.01$

three inbred lines are shown in Table 1 and the analysis of variance of the means is given in Table 2. The significant difference ($P < 0.01$) of the means among lines indicates the underlying line differences within Leghorn fowls (Table 2). The significant orthogonal contrast (Table 2) further dissects the possible differences in mean pock response to the virus for two down colour classes of embryos, supporting the hypothesis that black embryos of the W line are significantly ($P < 0.01$) more resistant to the virus than the white embryos of the I and C lines. Since the contrast 2 (Table 2) was not significant ($P > 0.05$), it may be concluded that white embryos of the I and C lines respond to virus in a similar way and that the response of the black embryos in the W line contributed more variance to the means and produced a significant line difference ($P < 0.01$) among the lines. These findings suggest that underlying differences among these isozygous lines may be due at least partly to genetic differences at the plumage colour locus.

Table 3. The *average genotypic/phenotypic value of pock counts in log scale for each subclass with respect to tumour virus and plumage colour loci, after inoculation of embryos with RSV(RAV49) (Experiment 2)*

Phenotype for tumour virus locus	Phenotype for plumage colour locus: White $I-$ (.9)	Black $i^+ i^+$ (.1)	$2a$ = effect at plumage colour locus
Susceptible $c^s -$ (.33)	$\bar{x} = 1.9994$	$\bar{x} = 1.8540$	0.1454
Resistant $c^r c^r$ (.67)	$\bar{x} = 0.0245$	$\bar{x} = 0.1527$	−0.1282
$2a$ = effect at tumour virus locus	1.9749	1.7013	

Table 4. *Analysis of variance for the four sub-class means described in Table 3 (Experiment 2)*

Source	df	SS	MS	F-value
Phenotypes	3	135.6112	45.2037**	1403.84**
Tumour virus C (*tvc*) genotype	1	135.3528	135.3528**	4203.50**
Plumage colour genotype	1	0.0016	0.0016	<1
Interaction	1	0.2568	0.2568**	7.97**
Error	158	5.0910	0.0322	

** $P < 0.01$

There may also exist an interaction (presumably an epistatic gene action in a quantitative scale) between the tumour virus locus c (*tvc*) and the plumage colour locus (I). This possibility was tested in the embryo population derived from W×R cross.

Embryo population derived from W×R cross: Experiment 2.

In Table 3 the mean phenotypic-genotypic pock count value in the log scale for each subclass has been presented within each cell. The difference in values are shown in the last row and column for the plumage colour locus and the tumour virus locus respectively.

It may be seen that the differences between genotypic mean values are not independent of the other

Table 5. *Observed and expected subclass phenotypes distributed within respective dilutions of*

Experiment Number	Dilution of virus	White susceptible: Observed frequency	Expected frequency	% observed	White resistant: Observed frequency	Expected frequency	% observed
2	$10^{-2.4}$	48	48.1	29.63	98	97.7	60.49
3	$10^{-1.4}$	57	55.1	53.27	41	43.3	38.32
	Difference in % subclass		−23.63			+22.17	

* ($P > 0.05$)

genotypes; the c^s gene has a larger effect on the *I*-genotype than on the $i^+ i^+$ genotype. Thus the two loci show epistatic interaction and do not combine additively. The analysis of variance for the subclass means is given in Table 4. The main effect due to tumour virus locus was highly significant ($P < 0.01$) in contrast to the main effect due to the plumage colour locus. Hence an existence of a direct role of the plumage colour gene for determining the tumour pock response on CAMs could be ruled out. However, since the effect of interaction between the tumour virus genes and the plumage colour genes was highly significant ($P < 0.01$), it could be concluded that there was non-additive combining of genotypic effects at the two loci for tumour pock response on CAMs. Hence it supports the hypothesis that the i^+ gene associated with the c^s-genotype partially inhibits the pock incidence on CAMs and thus decreases the susceptibility of the black embryos to the virus. The average pock count of the black susceptible embryos in the original scale was 73.20 as compared to 119.48 for the white susceptibles with a range of 46 pocks between the two means.

In this population the gene frequencies for i^+ and c^r were estimated to be 0.31 ± 0.02 and 0.82 ± 0.02 respectively. The c^r gene frequency estimated in this study by *in vivo* analysis was in perfect agreement with the estimate based on an *in vitro* tissue culture assay (Payne and Pani, 1971). Based on the estimates of gene frequency of the i^+ and c^r the observed subclass frequencies did not differ significantly from the expected subclass numbers ($P > 0.05$, Table 5). It is, therefore, assumed that the tumour virus genes and plumage colour genes were on different chromosomes because the frequencies of the subclass phenotypes observed in the fifth generation cross between the W and R lines were indicative of random recombination of the genes with an equilibrium of the respective genotypes (Lush, 1948).

Experiment 2 and Experiment 3.

The observed percentage of the four phenotypic subclasses and the estimated gene frequencies for i^+ and c^r are presented in Table 5. The gene frequency for i^+ estimated by maximum likelihood method based on proportion of resistant and susceptible CAMs in the experiments did not change when the virus input per embryo was increased ten-fold whereas the gene frequency for c^r (0.82 ± 0.02) at a virus dilution of $10^{-2.4}$ changes to 0.66 ± 0.02 at $10^{-1.4}$. This was not expected. The possibility of a sampling error was ruled out by appropriate statistical analysis. Furthermore, the χ^2 value computed for observed and expected subclass phenotypes within dilution of virus (using the respective gene frequency) did not differ significantly ($P > 0.05$) from the expected value (Table 5). The change in gene frequency cannot be therefore readily explained if the same gene is assumed to be expressed at different levels of virus input. This assumption is probably not true as a χ^2 analysis shows that the response of white embryos is contingent on the virus input whereas that of the blacks is not (Table 6). The white embryos are therefore more sensitive to high virus input than black embryos and this may explain the apparent change in the c^r gene frequency.

Discussion

It is clear that black embryos of the W line are more resistant than white embryos of the I and C lines to subgroup C virus. This is evident from the analysis of variance in which the mean pock count of the I or C inbred lines was significantly higher than that of the W line (Table 2). This difference may be partly accounted for by an inhibitory action of the i^+ gene on host susceptibility. In experiment 2 where four possible phenotypic variants had differences in the mean pock counts the range of about 46 pocks was observed between the two susceptible variants. Furthermore, there was a highly significant interaction between the two loci indicating a non-additive combining of the genotypic effects (Table 4). In experiment 3 the virus input per embryo was increased ten-fold and the pattern of segregation of resistant and susceptible classes of embryos within embryo down colour phenotype was measured. The expected proportionally equal rise and fall of the susceptible and resistant CAMs within respective down variants (white and black embryos) was not observed. Instead the ratio between the percentage of rise in susceptible embryos and the percentage of fall in resistant embryos within white embryo pheno-

RSV(RAV49) with the gene frequencies for c^r and i^+ in Experiment 2 and Experiment 3

Black susceptible			Black resistant			Gene frequency		χ^2-value
Observed frequency	Expected frequency	% observed	Observed frequency	Expected frequency	% observed	c^r	i^+	
5	5.4	3.09	11	10.8	6.79	0.82 ± 0.02	0.31 ± 0.02	0.056*
3	4.8	2.80	6	3.8	5.61	0.66 ± 0.02	0.29 ± 0.02	2.137*
	+0.29			+1.18				

Table 6. *The χ^2 analysis on the Expt. 2 and 3 for the segregation of resistant and susceptible subclass phenotypes when the virus input per embryo was increased tenfold*

	Sources	df	χ^2 value
Contrast (1)	white embryo Vs virus segregation input	1	15.30**
Contrast (2)	black embryo Vs virus segregation input	1	0.01
Contrast (3)	colour Vs virus segregation input	1	0.16
	Total	3	15.47**

** $P < 0.01$

type was 1.07 against the value of 0.24 for the black embryo phenotypes (Table 5).

These experiments therefore strongly suggest that the presence of the i^+ gene in the host genome has significantly modified the incidence of pocks on CAMs. However, the possibility cannot be ruled out that the modifying effect on host's susceptibility is due to another locus which is closely linked with the *I* locus. Further it was shown that the tumour virus genes and plumage colour genes segregated independently (Table 5).

At least two possible hypotheses can be put forward to explain the inhibitory influence of i^+ gene on the pock numbers:

1. A pre infection event:

It is possible that there are fewer cellular virus receptors coded for by black embryos as compared with white embryos. Hence reduced average pock incidence on CAMs of black embryos as compared to that of the white ones may be an outcome of reduced number of virus particles permitted to enter the cells. However, whether the reduced number of cellular virus receptors is an outcome of an interaction between i^+ gene and tumour virus gene cannot be speculated any further at this stage in the absence of additional information on the function of i^+ and the tumour virus gene in the host.

2. A post infection event:

The susceptibility to infection of cells from black and white embryos may be the same but oncogenic transformation of the infected cells may be influenced by the presence of the i^+ gene. Again, there is no information on how such an effect could be mediated.

There are no reports in the literature on the effect of plumage colour genes in chicken on either gross tumour incidence or cellular response to RSV. However, there is much evidence to support an influence of plumage colour genes on fundamental biological processes in the fowl. A body weight depressing effect (Jaap and Grime, 1956; Smith and Nordskog, 1963) and reduced embryonic viability (Collins and Hubbard, 1958) have been reported in chickens due to the dominant white (*I*) gene acting pleiotropically. Hamilton (1940) reported that the embryonic melanophores of both dominant and recessive white breeds of chicken are more sensitive to adverse environmental conditions and are more short-lived than the melanophores of breeds having black plumage. In mice, increased body size due to the blue gene, d (McArthur, 1949) and a significantly higher incidence of a reticular neoplasm due to A^y, yellow gene (Deringer, 1970) have also been reported. Therefore, it is not unlikely that the pigment inheritance system in chicken having black plumage may have an influence on the susceptibility to tumour viruses. The Leghorn fowl is a genetically coloured bird, its colour being suppressed by the inhibitor dominant white gene *I* present in the host genome. Possibly the *I* gene makes the genetically coloured bird more susceptible by increasing the optimal cellular environment for virus infection or replication.

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