

PROJECT JANUS
ANNUAL REPORT 1970-71

Although the major part of the work of Project JANUS at the Battelle Institut, Frankfurt, continues to be long-term mouse-skin painting experiments, the emphasis has moved some way towards the development of a short-term promotion test and the setting up of inhalation studies.

All the mice in the first seven long-term experiments are now dead but much of the detailed histology on the more recent experiments remains to be done. The presence of a virus infection in the mice supplied for experiment B10 caused much additional work early in 1971, and will probably delay the next long-term experiment.

During the year a considerable amount of time has been spent in developing a computer scheme for the validation and initial analysis of long-term results. The complete lack of documentation of the original program necessitated total revision of the scheme by outside consultants. This work, together with the essential acceptance tests, has now been completed. The data from experiments B0, B1 and part of B2 have been validated.

The hyperplasia test was used extensively as a routine short-term test during the first half of the year, but usage has declined latterly. This is partly due to interest in the sebaceous gland test, which has been developed in Hamburg and at Huntingdon, and also due to the work on a test for tumour-promoting activity.

There have been no staff changes in the JANUS team at Battelle.

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Dr. F.A. Sacherer is the administrative head of the toxicology section and Dr. J. Kiendl co-ordinates the JANUS project. Dr. E. Karbe is taking increasing responsibility for the scientific leadership of the project.

LONG-TERM TEST

During the year an R&DE report (RD 773) has been issued which included an analysis of the provisional JANUS results of experiments B0 to B7, excluding the single dose level experiments B1 and B5. In this report a number of assumptions had to be made and certain experiments simulated to obtain results in a form suitable for the mathematical analysis. The main conclusion of this report was that the dose levels used at Battelle were too high and should be reduced.

Based on this report with its inherent assumptions, and on the age-standardised results, the following comments on the initial experiments can be made.

Experiment B0 (150 weeks, all mice dead)

This experiment with smoke condensate from a flue-cured blend was undertaken to form a base line against which other materials could be compared. A satisfactory dose-response curve was not established and for this reason additional lower condensate levels have been incorporated into experiment B10.

A valid masterfile of the B0 data has been established and

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the initial tabulations of the data at MSD have been undertaken. The detailed statistical analysis is in progress.

Experiment B1 (136 weeks, all mice dead)

Using B0 cigarettes, the tumorigenicity of condensate produced at puff volumes of 10, 25 and 50 ml was examined. A valid masterfile of the data has been established at MSD Millbank, but this has not yet been analysed.

The preliminary analysis of this data indicates that the activity of the condensate is higher at low puff volumes.

Experiment B2 (160 weeks, all mice dead)

The smoke condensate from a "typical" US KSFT cigarette was examined. All the data from this experiment has been encoded and is currently being validated.

The provisional analysis indicated that the tumorigenicity of the B2 condensate was significantly lower than that of B0.

Experiment B3 (152 weeks, all mice dead)

This cigarette was manufactured from PCL (100%) produced in Montreal from CN102 lamina and Canadian flue-cured stem. The activity of the condensate is lower than that of any other cigarette examined at Battelle to date. The tumorigenicity, based on the provisional analysis, is significantly lower than that of cigarettes B0, B2 and B4.

Experiment B4 (152 weeks, all mice dead)

The cigarette used in this experiment was manufactured from equal proportions of flue-cured lamina and Canadian flue-cured stem (as CRS).

The preliminary analysis indicated that the tumorigenicity of

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the condensate is significantly lower than that of the B0 cigarette (flue-cured lamina) but only marginally lower than that of B2 (US blend).

Experiment B5 (148 weeks, all mice dead)

In this experiment, the condensates from cigarettes B0, B2 and B3 were re-examined to assess the reproducibility of the long-term tests. In addition, the effect of using a puffing frequency of three times per minute was also studied. In addition, following evidence of a change in the benzpyrene:tar ratio, the age-standardised results indicate that there is very good agreement between this experiment and the results obtained earlier. The change in puff frequency does not appear to affect the tumorigenicity of the condensate from the B0 cigarette.

Experiments B6/B7 (148 weeks, all mice dead)

These experiments were coupled so that the following effects could be examined using a flue-cured lamina blend:

- (a) Yeast treatment of the control tobacco.
- (b) Strand width (30, 60 and 120 cpi) using the control (B6) and yeast-treated tobacco (B7).

The provisional analysis indicates that the tumorigenicity of the condensates from B6 and B7 is significantly higher than that of B0. The reason for this is not known but results obtained by TRC show a similar increase in the activity of composite blends. Comparison of the results from B6 and B7 indicates that the yeast treatment has no significant effect on tumorigenicity. From the age-standardised results, it is apparent that the differences in strand width do not affect the tumorigenic activity of the condensate.

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Experiment B8 (week 100, in progress)

Six samples are being used to examine the effect on condensate tumorigenicity of an experimental PCL (X-PCL-5 ex Louisville) and a Celanese smoking material (I-308). Each of these materials is being examined on two levels of incorporation (60% and 100%). A silica gel filter attached to the control cigarette is also included.

At the present time, the predicted age-standardised results indicate that the incorporation of X-PCL-5 will lead to a reduced activity and that this reduction is related to the level of incorporation. The results for both samples which include I-308 indicate that the tumorigenicity of the condensate is very similar to that of the control flue-cured tobacco blend. This interim result is in agreement with the claims made by Celanese for this material: the delivery of condensate from these cigarettes is considerably lower than that from the control. The use of a high pressure drop silica gel filter, which reduces smoke delivery by about 40%, gives rise to condensate which currently is predicted to have a slightly higher tumorigenicity than that of the control tobacco.

Experiment B9 (week 56, in progress)

In this experiment, six samples are being used to study the effect of ethanol extraction, the Gerlach tobacco reconstitution process and the possible combination of these processes. Details of the samples were given in the last annual report, but it is still not possible to assess the results with a reasonable degree of confidence. In itself, this position indicates that the tumorigenicity of some of the condensates is likely to be low.

Experiment B10 (week 20, in progress)

This experiment is designed to examine the effect on condensate

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tumorigenicity of non-combustible filler (NCF) incorporated into the control flue-cured blend at 25% and 50% levels, the use of appreciable levels of carboxymethylcellulose as a binder, and to compare the activity of PCL directly with the control tobacco in a single experiment.

In view of the earlier findings, the dose level of condensate has been reduced and condensate from the control tobacco is being applied at five dose levels to establish a dose-response curve.

Animal Husbandry

Following various difficulties with ASL mice, the supplies over the last year, including those for experiments B9 and B10, have been obtained from Carworth (Europe) Ltd. As a result of an outbreak of chronic respiratory disease at Harrogate early in 1971, extensive checks have been made on mice received from Carworth, in particular those supplied immediately prior to and for the long-term skin-painting experiment B10.

From the preliminary checks and the appreciable death rate immediately following the cold shock treatment which is carried out soon after arrival, it was apparent that the mice for experiment B10 had been infected. Despite considerable efforts and help from the German State Veterinary Laboratories, the exact nature of the infection could not be diagnosed, although all the evidence indicated that the origin was a virus or mycoplasma. The suppliers claimed that they had no indication of a chronic infection until, four months after shipment, the presence of Sendai Virus was admitted.

The animals were given an additional stress in the form of a second cold shock and were then maintained in the quarantine room

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for a further four weeks. During this period, the health of the animals stabilised satisfactorily but it became apparent that the infection had been transmitted to the main animal house. The reasons for this transfer of infection from the quarantine room, despite its separate air circulation system, were diagnosed and two minor structural alterations have now been made to remedy the situation.

Apart from the additional work and delay in relation to other aspects of the project which this infection has caused, the presence of a virus infection will affect the start of the next long-term experiment. It is hoped, however, that Carworth (Europe) Ltd. will be able to supply mice of the same (CF/LP) strain but of improved quality from a new colony which is being established by hysterectomy derivation.

Analysis of Long-Term Experiments: JANUS Computer Scheme

As indicated in the introduction, this system has been completely revised and new programs written during the year. Although considerable delays have been experienced, the system has been fully documented and is now operating satisfactorily.

The main features of the system are the validation of the data, the updating of the masterfile, and the production of reports under four main headings: (1) tumour-bearing animals, (2) multiple tumours, (3) tumours outside the painted area, and (4) predominant pathology of all mice. The first two reports are in a form suitable for detailed statistical analysis and allow for the inclusion or exclusion of regressions and/or replacements. The tumour-bearing animal data is also copied to a magnetic tape file for transfer to

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a computer bureau and subsequent analysis.

It is already apparent that one of the advantages of the system is that all the data is submitted to a comprehensive validation routine. Early experience indicates that even after extensive manual checking, an appreciable number of errors are found. The facility for tabulating results in ad hoc groups is also proving valuable in the analysis of experiment B0.

SHORT-TERM TESTS

(a) Hyperplasia Test

During the first part of the year, over 30 samples were submitted for testing at Battelle, but the demand for this facility has declined sharply in the last six months.

Results received since the last annual report include the repeat test of B9 long-term sample, using Carworth mice. The results confirmed the previous hyperplasia test, which indicated the considerable reduction in activity resulting from manufacture into PCL or Gerlach sheet and an appreciable reduction after ethanol extraction of the tobacco. Further work on the reconstituted tobacco sheet materials showed that condensate from Burley stem sheet was significantly more active if the extract was discarded, but significantly less active if the extract was added back during manufacture. The activity of the condensates from cigarettes containing NCF was not significantly different from that of the control.

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(b) Promotion Test (Skin-Printing)

An initial test has been undertaken to estimate the promoting activity of condensate from samples B9-1 (control tobacco) and B9-6 (tobacco extracted with ethanol and manufactured into Gerlach sheet). In this study the mice were pre-treated with the carcinogenic initiator (dimethylbenzanthracene) applied at two dose levels. Promising results were obtained and these are being followed up in a more comprehensive experiment. The initial results indicate that the promoting activity of the B9-6 condensate is very much less than that of the control (B9-1).

(c) Sebaceous Gland Test

Facilities for this test are available at the B-AT laboratories in Hamburg and also through the courtesy of the Imperial Tobacco Group Ltd. at Huntingdon Research Centre. Arrangements for these tests are now being organised on a very similar basis to the hyperplasia tests at Battelle and a brief review is included with this review of the main JANUS programme.

Initially some of the JANUS long-term test samples were examined independently at Hamburg and Huntingdon. It was found that the Hamburg results correlated very well with those obtained previously in long-term experiments, and also in the short-term hyperplasia test. In contrast, the correlation of the Huntingdon results with these tests was poor. Various improvements have been made at Huntingdon and a preliminary assessment of a recent collaborative test between the laboratories indicates that the results are very comparable.

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GENERAL

Although mouse-skin painting is likely to remain an important recognised test of carcinogenesis for some considerable time to come, the emphasis is changing and more importance is being placed on assessing materials using a variety of techniques. This is particularly true when considering new materials. For tobacco smoke the emphasis on inhalation tests has still not resulted in the development of a routine chronic technique, still less on an acceptable method of test.

It is hoped that our own laboratory facilities will be set up during the coming year. When these become available, the programme will initially be concentrated on developing inhalation techniques with special attention to the accurate measurement of the dose retained in the respiratory tract. Measurement and control of the dose retained from different cigarettes is an essential prerequisite for comparing results from inhalation experiments.

With this background, it is anticipated that mouse-skin painting experiments will be continued at Battelle and although the demands are reducing the next long-term test has already been planned. Future requirements are somewhat less certain but it is likely that such tests will be required for the examination of new technical developments in the reconstitution process and the incorporation of different materials or additives in the process.

Further efforts will be concentrated on the development of reliable short- or medium-term tests using both skin-painting and inhalation techniques. It is anticipated that the promotion test

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(skin-painting) will provide a reliable means of assessing smoke condensate. Although this test should make it practicable to assess promotion activity within a six-month period, it is possible that a longer test period will be required for condensates of low activity.

The sebaceous gland test is favoured at present, compared with the hyperplasia test, although both correlate well with long-term results despite some anomalies apparent in the results from both procedures. It is expected that requirements for hyperplasia tests will continue at the present much reduced level with slightly more interest being shown in the sebaceous gland test.

The promoting activity and the possible mutagenic effect of smoke will be assessed in two inhalation experiments due to start early in the year. The main objective is to obtain first-hand experience of inhalation techniques with mice and hamsters. It is naturally hoped that these experiments will lead the way to the further development of inhalation tests, and also provide some initial results.

LONG-TERM TEST

A new long-term skin-painting experiment has been planned to compare, within a single test, the effect of reconstituting tobacco via the PCL and the paper (Schweitzer) processes.

This experiment was planned to start early in 1972 but, following initial delays in obtaining facilities at Le Mans, the

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start is likely to be further delayed due to the respiratory virus infection in the main animal house. On a long-term basis, it is logical to delay this experiment until the animal house can be thoroughly disinfected and clean stock obtained.

SHORT-TERM TESTS

(a) Hyperplasia Test

Due to the large number of tests submitted early in the year and the additional work involved following the infection of the B10 animals, the results of a number of tests are still outstanding. Although the test will be continued, it is anticipated that the number of samples submitted will be small.

(b) Promotion Test (Skin-Painting)

A further experiment is due to start early in October. Samples B9-1 and B9-6 will be re-examined to assess the reproducibility of the test and to examine the effect of continuing treatment past 30 weeks. The experiment has been designed on a statistical basis so that the effect of DMBA and condensate dose level and any interactions can be determined. The four remaining samples from the B9 series will be examined in the same experiment. Further work will be dependent on the results of this test.

(c) Sebaceous Gland Test

Requests for tests on a number of samples have already been received from Montreal and Louisville. A further series of JANUS samples will also be considered so that the relation between the

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sebaceous gland test and promoting activity can be examined.

INHALATION TESTS

(a) Promotion Test

In this test, which will take approximately one year to complete, N-methyl-N-nitrosourea (MNU) will be applied topically to the hamster larynx prior to exposure in a Hamburg type inhalation apparatus. Exposure will continue for six months at which stage the animals will be sacrificed and serial sections of the respiratory tract examined for pathological changes.

(b) Mutagenesis Test

There is an increasing awareness of the importance of tests which assess toxic materials in terms of their effects of genetic processes. In this test, which is of a completely exploratory nature, male mice will be exposed to smoke aerosol. Subsequently they will be mated and the females sacrificed prior to parturition. The number of pregnant females, corpora lutea, live and dead implants will be counted and the results assessed by the Dominant Lethal Method.

Two JANUS cigarette samples will be examined in this test which is due to start in October.

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REPORTS

- B19 : Comparative investigations on several carcinogens with regard to their proliferation activity in the short-term experiment and their tumour provocation effect in the limited long-term experiment.
- B20 : Problems associated with the infection of ASL mice.
- B21 : Suppression of non-specific esterase activity in mouse skin sebaceous gland by tobacco condensates.
- B22 : Assessment of acute upper airways irritancy of a range of cigarettes.

SRE/SEW/3.5

13th October, 1971

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