SCREENING DATA FROM THE CANCER CHEMOTHERAPY NATIONAL SERVICE CENTER SCREENING LABORATORIES. XXXIV. PLANT EXTRACTS

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SUMMARY

Data are reported on 1457 plant extracts which were tested in the primary screens of the Cancer Chemotherapy National Service Center. Almost all the materials were tested in Sarcoma 180, Adenocarcinoma 755, Leukemia 1210, and KB cells in culture; some tests were also carried out in Friend virus leukemia, Lewis lung carcinoma, Human sarcoma HS1, Adenocarcinoma of the duodenum, and Melanotic melanoma. Data are reported only on materials that have not demonstrated sufficient activity in these systems to warrant further investigation.

INTRODUCTION

This report is a continuation of the series of publications of Cancer Chemotherapy National Service Center (CCNSC) screening data, and is the seventh report dealing with plant extracts. Data are included on systems incorporated into the primary screening spectrum a few years ago as well as those used exclusively until that time. The presentation of data on these systems has been made possible by the reduction of all information to a unified line summary, which facilitates data storage and retrieval by an IBM 1401 computer. A substantial change in data presentation was made recently to permit the inclusion of the newer tumor systems.

The following description of materials, procedures, and results is similar to that of earlier publications, which discussed preparation of plant extracts (17) and which first discussed the inclusion of the new test systems (18).

MATERIALS AND METHODS

Service Screening Laboratories

All the test data reported were obtained under contracts with the CCNSC. Test procedures are prescribed in a set of protocols followed by all contractors. Reports of the experiments are submitted to the CCNSC for review and statistical evaluation. The summary line for each experiment gives the code identification of the screening laboratory; these codes are listed later.

Source of Plant Extracts

Plant materials in Table 1 were collected, identified, and extracted under the auspices of the College of Pharmacy, University of Arizona. Most of the support for this work was by contract funds from the CCNSC, with additional support furnished to the College of Pharmacy by the Arizona Division of the American Cancer Society, The Elsa U. Pardee Foundation and University of Arizona Alumni Fund. Table 2 lists plant extracts prepared at the College of Pharmacy, Ohio State University. Plant materials used were in part obtained by the University and, in part supplied to the College of Pharmacy by the New Crops Research Branch of the U. S. Department of Agriculture (USDA) from collections made under a transfer of funds from CCNSC.

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In Vivo Procedures

Tumors and Host Animals. Almost all the materials have been screened in 3 transplantable mouse tumors: Sarcoma 180 (S180), Mammary adenocarcinoma 755 (Ca755), and an ascitic form of Leukemia 1210 (LI210). These are all well-known transplantable tumors and are adequately described elsewhere (5, 14, 16). The selection of these tumors by the Screening Panel of the CCNSC was based on the experience available in 1955, with great weight placed on the ability of these screens to select almost all the materials then considered clinically useful in man.

It was also recognized that these test systems may not be the optimal ones to predict for clinical effectiveness. Considerable effort was made to develop and evaluate a variety of additional systems that are different from the original ones in their drug sensitivity and can be used as reliable screens from the standpoint of reproducibility. As a result of this search, which is continuing, a large number of systems in mice, rats, hamsters, and embryonated eggs have been incorporated into the screen. The ultimate test for these systems will be the predictability, if any, for human cancer. As new agents are selected and tested clinically, it should be possible to discard the less useful systems and emphasize the more useful ones.

A new material of unknown activity is tested in 3 in vivo systems, selected on a random basis. If quantities are limited, fewer test systems may be used.

The test systems which are or have been used as primary screens, along with the host used for propagation and test, are as follows:

Tumor	Host for Preparation	Host for Test
Adenocarcinoma 755	C57BL/6 female mouse	BDF_1 mouse (C57BL/6 female x DBA/2 male)
Cloudman melanoma (S91)	DBA/2 mouse	BDF_1 mouse
Ehrlich ascites	Randombred albino mouse	Randombred albino mouse
Friend virus leukemia	DBA/2 mouse	BDF_1 mouse
Hepatoma 129	C3H/He mouse	C3H/He (or hybrid) mouse
Lewis lung carcinoma	C57BL/6 mouse	BDF_1 mouse
Lymphoid leukemia 1210	DBA/2 mouse	BDF_1 mouse
Osteogenic sarcoma HE10734	C3Hf mouse	C3H/He mouse
Sarcoma 180	Randombred albino mouse	Randombred albino mouse
Dunning leukemia ascites	Fischer/344 rat	Fischer/344 rat
Human sarcoma HS1	Suitable randombred rat	Suitable randombred rat
Murphy-Sturm lymphosarcoma	Suitable randombred rat	Suitable randombred rat
Walker 256 carcinosarcoma (intramuscular)	Suitable randombred rat	Suitable randombred rat
Adenocarcinoma of duodenum	Hamster	Hamster
Adenocarcinoma of endometrium	Hamster	Hamster
Adenocarcinoma of small bowel	Hamster	Hamster
Melanotic melanoma	Hamster	Hamster
Human sarcoma HS1	Embryonated egg	Embryonated egg

Preparation of Plant Materials. Currently a single aqueous/ethanol extract is made of each plant sample, but for the samples reported here 2 extracts were generally prepared, according to the following standard procedure.

Extracts listed in Table 1 were prepared as follows: Aqueous extracts were made by adding boiling distilled water to the ground plant sample (approximately 4 ml/gm) and allowing this mixture to stand at room temperature for 3 hours. The extract was decanted through a cotton-gauze filter, the filtrate reduced in evaporating dishes on a steam bath, and lyophilized.

The ethanol/chloroform extracts were prepared by adding equal parts of ethyl alcohol (95%) and chloroform to the ground plant material which was stirred intermittently for 24 hours. After filtration through a cotton-gauze filter, the filtrate was evaporated with air blowers at room temperature to a soft consistency which was then kept frozen until tested.

The extracts in Table 2 were prepared as follows: Aqueous extracts were made by stirring 100 gm of plant material in 1000 ml of distilled water for four hours. This material was filtered through glass wool and the filtrate reduced to a dry powder using a Stokes Freeze-Drier.

In the case of the ethanol extracts, a 100 gm sample of plant material was extracted with 95% ethanol using a Soxhlet extractor. After filtering, the extract was concentrated to a semisolid consistency in vacuo using a rotary vacuum concentrator.

The lyophilized aqueous extracts were dissolved in saline or suspended in 0.5% methylcellulose (MC) or carboxymethylcellulose (CMC). Ethanol/chloroform and other extracts were dissolved in a small quantity of 95% ethanol and then suspended in MC or CMC. Occasionally extracts were diluted with saline or with MC solution to a concentration not exceeding 2% alcohol.

Because of the gummy nature of these extracts, satisfactory suspension of the material was not always possible. Also marked changes in toxicity occasionally occurred when there was a long interval between successive tests. These facts must be considered in evaluating the data.

Test Procedures. The detailed protocols for each of the tumor systems listed previously have been published (22). Copies of the protocols are available upon request.[6] These protocols describe the methods currently in use. A previous report in this series (19) presents some of the earlier procedures.

Transplantation of tumors is carried out with aseptic procedures, including sterilized instruments and hooded areas. In every experiment fragments or suspensions are tested for sterility by putting samples into tubes containing thioglycollate broth. If bacterial growth occurs within 48 hr, the entire experiment is discarded.

The treatment procedure for each test is now presented with each line of summary data (see Results). In general one injection of the material is given each day intraperitoneally. The number of days of treatment varies with the test system.

Solid tumors are evaluated by weight, which is reported in grams for rat tumors and milligrams for all others. The ratio of the mean weight of tumors in treated animals to that in controls (T/C x 100 = x %) and the body weight changes of treated and control animals are recorded.

For L1210 the mean survival time of animals is calculated. A T/C value for L1210 tests is the mean survival time (in days) of the test group divided by the mean survival time of the control group, expressed as a percent. Median survival times are used for the Dunning leukemia system.

Deaths are recorded in all groups. A maximum tolerated dose for a single experiment is defined as the highest dose which produces 2 or fewer deaths among 6 animals or 3 or fewer deaths among 10 animals.[7] In L1210 and the Dunning ascites, deaths before Day 6 are considered nonleukemic and form the basis for toxicity evaluations. When a toxic result (more than 2/6 or 3/10 deaths) is observed, the test is repeated at an appropriately lower dose until the maximum tolerated dose is reached. In addition if the T/C value for survival tests is less than 85%, the dose is considered too high and is reduced.

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[7] In these tumor studies, the maximum tolerated dose is considered to be the LD_{10}, which is determined once a sufficient number of tests are carried out. On a single test with 6 or 10 animals, however, one cannot differentiate 2/6 or 3/10 deaths from the LD_{10}. These limits (2/6 or 3/10) are therefore used as the upper limits of lethality on a single experiment.

Control of the quality of the tumor systems in all laboratories is aided by the establishment of limits for deaths, "no-takes," and mean tumor weight (or survival time) among control animals. Control deaths exceeding 10% are considered excessive. Experiments in which the control animals fall outside of limits are evaluated and some or all tests are repeated as deemed necessary.

Additional quality control is imposed by the inclusion of a "positive" control substance as 1 test on at least every other control group. Tests of such materials thus amount to about 2% of all tests performed. The positive controls used for each test system are listed in the protocols (22). A dose of the positive control producing a substantial but not overwhelming response has been selected. This is done to enable one to see changes in susceptibility of tumor lines to the drugs, as well as to detect any technical errors which might occur.

In addition to the quality control measures just described, other efforts are made to standardize the test results among all laboratories. Among the procedures instituted to help standardize the tumors is a frozen tumor bank. A characterized, reliable line of each tumor is distributed from the bank to all screening laboratories at defined intervals. Thus the genetic drift is minimized and results at different laboratories should be more reproducible.

Cell Culture Procedures

Cell Lines. The cell line used for routine screening is KB (Eagle), derived from a carcinoma of the nasopharynx (9). This cell line was selected because of its rapid and reproducible growth rate as a monolayer culture. The work of Eagle and Foley in testing the cytotoxicity of a number of compounds against several cell lines indicates that all cells grown in monolayer culture behave similarly in their response to drugs (11,12). When the CCNSC cell culture program first started, all compounds were tested against Chang's liver (4) and some against HeLa (15) in addition to KB. An analysis of the data on more than 1000 compounds demonstrated that less difference in response occurred between simultaneous tests with 2 cell lines than between successive tests with 1 cell line. Thus it was decided to use one line, KB, for all routine tests.

Stock cultures on glass are cultivated on Eagle's basal medium (8) plus 10% calf, human, or any other suitable serum. The cells are maintained in a state of rapid growth by frequent subculture, generally every 3 or 4 days. The cultures are refed 24 hr before use on test.

Test Procedures. The original procedure was similar to that described by Eagle and Foley (11). Cells were removed from glass either by washing with trypsin or Versene or by mechanical scraping. Dispersed cells were centrifuged and resuspended in complete medium, or diluted to an inactive concentration of dispersing agent. About 50,000 cells in 1 ml of medium were implanted in a series of 15-mm screw-cap culture tubes; the tubes were placed at a 10° angle and incubated at 37° C. After 24 hr the original medium was removed and fresh medium containing the drug was added. The cultures were refed at 72 hr and the protein content was determined 1 or 2 days thereafter according to the method of Oyama and Eagle (21).

A simplification of this procedure was described by Smith et al. (26); the simplification or a modification of the procedure has been used since 1959. The suspension of cells is diluted to a concentration of 10-20 μg of cell protein per ml. Immediately after 0.1 ml of drug solution or suitable control material is put into each culture tube, about 3.9 ml of the cell suspension is added. The test is ended after 72 hr. Thus this procedure provides a more rapid test and eliminates the need for intermediate feeding of the cultures. A modification, which is used in some experiments, provides a 24-hr period for the cells to attach before the drug is added with fresh medium.

All of these methods give equivalent results, the only critical factor being the degree of cell multiplication. An experiment is considered satisfactory if the final cell protein is at least 6 times the initial amount.

Plant extracts are not sterilized but are handled aseptically. They are dissolved in water or saline, but if the necessary concentration cannot be obtained, ethanol, dimethylformamide, and other solvents are used. In such cases the final concentration of solvent is less than the amount known to affect the cell growth (27).

Routine testing of compounds is carried out at 100, 10, and 1 μg/ml. A material with an ED_{50} of less than 1 μg/ml (see calculations described later) is retested at lower concentrations; one with an ED_{50} above 100 μg/ml is considered inactive and is not retested. In some cases 5 doses at closer intervals are used when a more precise end point is desired.

The determination of cytotoxicity is based on the inhibition of cell protein synthesis. Measurements include the initial protein per tube (C_0), the final protein in control tubes (C), and the final protein in drug-treated tubes (T). The initial protein is determined on an aliquot of the inoculum or by using a series of control tubes at the time of drug addition. Internal controls include a protein standard (bovine serum albumin) and a group of tubes containing medium but no cells. The latter are needed because it was found that 20-30 μg of protein from the medium adhere to each glass tube. The equivalent of a T/C value, in this case $(T-C_0)/(C-C_0)$, is determined for each dose. It is assumed that this response varies linearly with the log of the concentration and is a straight line within defined limits of response (plotting a considerable amount of data has shown this to be true). A computer program for the IBM 1401 has been developed to calculate an estimated ED_{50}, the dose which inhibits protein synthesis to 50% of controls. A slope is also calculated, representing the change in response for a 10-fold change in concentration.

Approximately 50-75 materials (n) are tested simultaneously at 3-5 doses against a single group of controls. Each dose is run in duplicate tubes, and the number of controls is equal to $2\sqrt{n}$.

EXPERIMENTAL DESIGN

In Vivo Systems

Establishment of Test and Control Groups. In developing each of the test systems used in the primary CCNSC screen, the relative reproducibility of each system was used to establish reasonable test and control groups. Earlier studies had shown that satisfactory reproducibility at a reasonable cost required 10 animals each in treated and control groups for S180 and L1210, and 16 animals each for treated and controls with Ca755.

It has been shown, in testing many materials simultaneously against a single control (13), that an optimum use of animals can be made if (a) the number of control animals is increased by the square root of the number of treatments to be compared with that control, and (b) the sum of the reciprocals of the number of animals in the treated group and the control group is equal to what a simple treatment-control comparison would give, i.e., equal to (1/10 + 1/10) for S180 and L1210, and (1/16 + 1/16) for Ca755. Thus in the usual S180 experiment with 25 materials tested against a single control, there are 6 animals per test and $6\sqrt{25} = 30$ animals for the control group (1/6 + 1/30 = 1/10 + 1/10). A similar experiment for Ca755 uses 10 animals per treatment and 43 per control (1/10 + 1/43 = 1/16 + 1/16). For simplicity, new tumor systems were assigned to either the S180 design or the Ca755 design, depending on their relative reproducibility. Thus the Lewis lung carcinoma uses 6 animals per test while S91 uses 10.

The animals are assigned to the control and experimental groups by acceptable randomization techniques. Various randomization procedures are used, but all are based on standard published tables of random numbers such as the Kendall-Smith tables or the Rand Corporation "Million Random Digits."

Selection of Materials. The in vivo screening system used by the CCNSC was designed to select, for further examination, materials that have more than a specified minimum antitumor activity in at least 1 animal tumor system. Without prior knowledge of the true activity of materials presented to the screen, it was not possible to set up a screening mechanism which would select a fixed percentage of active materials from all those tested. By establishing minimum standards of biologic activity, and examining the earlier experience of screeners who had used the tumors CCNSC now uses, estimates were made of how many materials would be declared suitable for additional study. The preliminary calculations showed that each tumor would select for further examination 1-5% of the materials submitted.

To accomplish this rate of selection for the original solid tumors, S180 and Ca755, a multistage system was established for synthetic compounds. Based on the early work of Wald (28,29) in sequential analysis and, in part, some recent work of Armitage (1,2), a scheme was developed resembling the one used by Lederle Laboratories (designed by Charles Dunnett) (6,7). Detailed descriptions are given by Armitage and Schneiderman (3) and Schneiderman (24). A material passes the 1st test if it produces a T/C of less than 54%. It is then retested at the same dose. The product of the T/C values (expressed as a decimal) for the 1st 2 tests must be less than 0.20 or the material is rejected. If the material passes the 2d stage, it is tested a 3d time. For a material to pass the third stage, the product of the T/C values for the 3 tests must be less than 0.08. As a result of the sequential system, most work is done on the most active materials. A clearly inactive material is rejected after only 1 test. The borderline material, which has passed the low hurdle of T/C = 0.54, will usually fail on the 2d trial, which requires a (geometric) mean T/C of 0.45 (i.e., 55% inhibition as opposed to only 46% inhibition). The 3d trial imposes a still tougher limit by requiring an average T/C of 0.42 (58% inhibition) for all 3 trials. Truly active synthetic materials will pass the 3 trials and then a minimum of 3 confirmation tests. Based on this experience with synthetic compounds, a 2-stage sequential scheme was introduced for plant materials. Activity of a material passing the sequential test is confirmed by multidose experiments done with 2 extracts of the plant material.

As more solid tumor systems were added to the screen, it was apparent that different sequential schemes would be necessary, depending on the reproducibility and sensitivity of the system. For simplicity, all systems were assigned to 1 of 2 schemes, either the one used for S180 and Ca755 (Type 1) or a somewhat more liberal scheme (Type 2):

SCHEME

Type 1	Type 2
Stage 1: T/C ≦ 0.44	Stage 1: T/C ≦ 0.60
Stage 2: T/C ≦ 0.19	Stage 2: T/C ≦ 0.22

TEST SYSTEM ASSIGNED TO EACH SCHEME

Adenocarcinoma 755	Cloudman melanoma (S91)
Ehrlich ascites	Solid Friend virus leukemia
Hepatoma 129	Human sarcoma HS1 (rat)
Lewis lung carcinoma	Murphy-Sturm lymphosarcoma
Osteogenic sarcoma HE10734	Walker 256 carcinosarcoma (intramuscular)
Sarcoma 180	Adenocarcinoma of the small bowel
Adenocarcinoma of the duodenum	Melanotic melanoma
Adenocarcinoma of the endometrium	Human sarcoma HS1 (embryonated egg)

Note that these acceptance levels far exceed those required for a significance test such as the "t" test, which is often used. A significance test has been considered a necessary but not sufficien criterion for acceptance, since it accounts only for the variation among test objects run simultaneously Detailed statistical analysis of CCNSC data indicate that the variation within a test is exceeded by th variation between tests run at different times. In such cases a within-experiment significance test mus not be used.

The operating characteristics of these screens do not provide a sharp distinction between "active and "inactive". Therefore the activity of plant extracts passing the 2-stage sequence must be confirmed Fresh extracts of the plant material are tested at 4 doses, 1 the same as the sequential dose, 1 higher and 2 lower. The S180 and Ca755 doses are about 0.2 and 0.3 log apart, respectively. Two dose-respons experiments are normally done with 2 different extracts. To be confirmed in most solid tumors, a materi must show reproducible activity (T/C ≦ 42% for S180) at a nontoxic dose. For a material to be confirme in Ca755, a reproducible therapeutic index of 2 is necessary; the index is $LD_{10}/(T/C_{42\%})$. A reproducibl T/C of <10% at a nontoxic dose is also sufficient for confirmation. Host weight loss, as described late is considered in establishing the nontoxic dose at this stage.

The L1210 and Dunning ascites system is a 2-stage sequential scheme. The minimum T/C value for the 1st test is 125%, which represents an increase of 25% in the mean (median for Dunning ascites) survival time of test animals over controls. To pass the 2d test, a material must produce an effect such that the product of the T/C values of the 2 tests (expressed as a decimal) is ≥ 1.56. The confirmation procedure is the same as that for solid tumors; the doses are 0.2 log apart and a material is confirmed if at least 1 dose from 2 extraction samples produces a T/C $\geq$ 125%.

Extracts which pass the 2-stage sequential test and meet the criteria in the confirmation test are considered "active" and fractionation and isolation work are recommended.

Up to this stage of testing, no effects of toxicity (other than death) are considered in evaluating the compounds. Since it is well known that the growth of many tumors is influenced by weight loss, its effect is evaluated for compounds passing the confirmation procedure. The difference in weight change between the treated and control animals is considered rather than the weight change of the treated animals alone.

Despite intensive efforts, it has not yet been possible to develop a completely general solution to the influence of weight change (or weight loss) on tumor growth, even though all solid tumors are affected. As an interim measure, specific weight change differences for each solid tumor system are considered evidence of toxicity. Prior experience of CCNSC and other investigators with these tumors was considered in establishing these criteria (22). Recently Skipper et al. (25), reporting on the use of a "specificity test," indicated that a method may now be available for determining this effect more definitively. Since the method does not draw a conclusion for each test but rather uses all data to draw a general conclusion for a given test material, it tends to minimize the variation in this phenomenon from one experiment to another. This method is now used in the evaluation of all compounds at the confirmation stage.

Performance Characteristics. The major concerns one has with a screening system are its reproducibility and its stability. The standard deviation of the T/C from experiment to experiment is the most meaningful measure of reproducibility. For the sample sizes used in these studies, the "between-test" standard deviation of the T/C values is 0.13 (in log units) for both S180 and Ca755. The sample sizes for the S180 and Ca755 were adjusted to their present levels to equalize the between-test standard deviations. A log standard deviation of 0.13 means this: given a T/C on a material, one would expect that the next trial of this material would give a T/C (95% of the time) between $(T/C)_1/1.82$ and $(T/C)_1 \times 1.82$. For example, if the 1st T/C is 60%, one should not be surprised to see a 2d T/C on this material anywhere between 33% and 109%. The log standard deviation for the new systems has ranged from about 0.11 to 0.25. It was on the basis of this standard deviation that the experimental groups and sequential schemes were assigned. CCNSC results and those of other investigators using these tumor systems have the same order of reproducibility.

The variation in T/C from test to test is a result of the inherent variability of the animal systems. Two successive T/C values which are close to each other are not the result of unusual reproducibility of the material, but are merely a chance occurrence, whose frequency could be readily computed. To ascribe any special qualities to a material because of a pair of closely similar T/C values is to misread the results of the animal screen.

Cell Culture

Selection of Materials. Cell culture testing originally began when a random sample of synthetic compounds was tested in the CCNSC rodent tumor screens to determine the correlation between cytotoxicity and activity in one of the tumor systems. This was an extension of the work of Eagle and Foley (10). Data on almost 2000 compounds (23) showed that a good correlation exists and that the correlation improves as cytotoxicity increases. Therefore it was decided to test plant extracts in the cell culture system as a primary screen along with 3 in vivo systems. It has also been used specifically for testing materials available only in small quantities and selecting those that should be prepared in larger quantities for in vivo testing.

The degree of cytotoxicity required depends on the purpose for which the information will be used. As mentioned, there are several possible uses for the cell-culture test and thus several different end points.

In the study of plant extracts, the cell culture test is used for selecting materials to be isolated and further evaluated in vivo; the test is set to choose 10% of the compounds. The 10% end point, which enhances the expectation of in vivo activity (about 20% probability), is 20 μg/ml. A 2-stage testing system was chosen. A material passes the 1st stage if the ED_{50} is ≦ 30 μg/ml. To pass the 2d stage, a compound must have an arithmetic mean from the 1st 2 tests of ≦ 20 μg/ml. A material passing the 2d stage is confirmed by a 2d extraction of plant material.

Performance Characteristics. An estimate of the reproducibility of the ED_{50} was made, based on tests of a positive control compound at 4 laboratories; the number of replicates at each laboratory ranged from 10 to 47. Since the ED_{50} is a logarithmic function, as mentioned earlier, the standard deviation of the log ED_{50} was calculated and was 0.208. This value means that, given an ED_{50} (A) on a material, one would expect (95% of the time) to obtain a value between A/2.61 and A x 2.61. These tests were carried out at 5 doses at 0.3 log intervals. The reproducibility under these conditions is substantially better than that previously calculated on the basis of tests with 3 doses at 1 log intervals (20).

RESULTS

Data on plant extracts which failed the CCNSC primary screens or confirmation tests are presented. In general the materials were tested in 3 rodent tumor systems and cell culture. The in vivo data include 1 toxic result (if any toxicity occurred) as an aid in determining dosage for other investigators using these materials.

The format of the data summary has been revised to provide additional information, including route of administration, vehicle, day of first injection (day of tumor implantation is defined as Day 0), frequency of injection, total number of injections, and day of sacrifice. The addition of this information permits the publication of data on all systems now used as CCNSC primary screens. It will also facilitate the publication of data on active compounds, since variations in route, frequency, etc. can now be included as normal computer output. The experiment number, also now included, will aid in the evaluation of the data from the chronologic standpoint. Codes used in this report are given, as well as some additional codes which will appear in future reports.

The cell culture results are presented in a new format as an "on-line" print rather than in fixed columns, since these data represent a minor part of the total information reported. This change provides the space needed for the additional information mentioned.