Beryllium Carcinogenesis

II. Pulmonary Deposition and Clearance of Inhaled Beryllium Sulfate in the Rat

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SUMMARY

The pulmonary beryllium levels of rats exposed to the inhalation of BeSO₄ aerosol at a concentration productive of lung cancer in 100% of the animals (34.25 µg of Be/cu m) showed a rate of accumulation which decreased during continuing exposure. After about 36 weeks, a tendency toward a concentration plateau was apparent in both sexes. The plateau is interpreted as equilibrium between deposition and clearance; mechanisms of the latter are shown to include not only the solubility of intrapulmonary precipitates formed in situ but also certain host-dependent factors involving primarily the lymphatic route. Females were distinctly less efficient than males in utilizing this route of clearance, resulting in slower removal of pulmonary Be deposits, markedly lower accumulation of the inhaled material in the regional lymph nodes, and earlier morbidity and mortality. Tracheobronchial lymph node loads reached peak values concurrently with the attainment of the pulmonary plateaus and showed a decrease from the 52nd week. This is interpretable as diminishing accumulation due to progressive impairment of the normal clearance routes during the exposure and as increasing rate of systemic dissemination from the lymph nodes with higher beryllium loads. Only about one-half of the original pulmonary load was cleared rapidly; the remainder tended to remain in the lungs for longer periods and probably become incorporated into the nuclei of certain pulmonary cells. This portion of the inhaled beryllium appears to be involved in the exertion of the carcinogenic challenge.

INTRODUCTION

In the 1st article of this series (20) we reported on the production of pulmonary adenocarcinoma in the Sprague-Dawley (C.D.) rat by inhalation exposure to a microaerosol of beryllium sulfate at a mean atmospheric concentration of 34.25 µg of Be/cu m and a mean particle size of 0.118 µ in diameter. Exposure was provided 7 hr daily on weekdays, totaling 2400 hr in the course of 72 weeks. Monthly sacrifices showed a progressive inflammatory as well as a proliferative response. The 1st tumors appeared after 9 months; thereafter the incidence rapidly rose and reached 100% at 13 months (versus 0% in controls).

Beryllium assays were performed on the lungs, tracheobronchial lymph nodes, and blood of these animals at the time of their sacrifices. These assays are reported in the present article, with discussion on the turnover of inhaled beryllium in the bronchopulmonary tissue of the rat undergoing carcinogenesis.

MATERIALS AND METHODS

Animal exposure and sacrifice methods were discussed previously (20). The exsanguinated, excised, and blotted lung lobes were subdivided according to the regions of gross pathology, and weighed aliquots were obtained from each specimen for histopathologic and biochemical studies. The remainder of each specimen, comprising as large a part of the tissue samples as possible under the experimental conditions (average, about 1/3), was submitted to spectrographic beryllium analysis. The analytic results were calculated in terms of the whole lungs, assuming uniform distribution of beryllium within each pathologic region. The tracheobronchial lymph nodes were excised, weighed, and similarly analyzed in toto. Blood was obtained from the right atrium in an average amount of 5 ml/animal and preserved with 0.1% heparin.

Beryllium analyses were performed by a modification of the method of Smith et al. (26), using the a.c. spark, and measuring the λ = 3130.4 Å beryllium line (for concentrations greater than 5 µg of Be/ml, the λ = 3131.2 Å beryllium line) in conjunction with the λ = 3059.9 Å aluminum internal standard line. Modifications of the original method included the use of porous graphite cup upper and shaped graphite rod lower electrodes in place of the rotating electrode assembly; elimination of the ethylenediaminetetraacetate-MnCl₂ separation and coprecipitation procedure except for specimens over 1.0 gm of dry weight; and the selection of electrical and optical settings best suited to the available instrument (Table 1).

The samples were dried to constant weight, ashed at 550°C, and reweighed. The residues were digested in a 3:1 mixture of concentrated HNO₃:HClO₃ in order to convert all beryllium into a soluble form. One to 5 ml of a 0.1% AlCl₃ solution was then added to serve as an internal standard (this quantity is sufficiently large in comparison with the aluminum content of the specimens to render any variations in the latter negligible), and the solutions were assayed spectrographically.

Sensitivity of the method reached 0.005 µg of Be, with an accuracy of ±10%.

Acid and alkaline phosphatases were assayed by the auto-
RESULTS

Pulmonary beryllium was present in all specimens derived from exposed animals, with the amounts gradually increasing during exposure (Chart 1). Males, because of their larger size, accumulated greater average amounts than females, but in both sexes the rate of accumulation decreased during continuing inhalation. A tendency toward a concentration plateau was apparent in the lungs of rats sacrificed after about 36 weeks of exposure, indicating that the animals tended toward the establishment of an equilibrium between deposition and clearance after an initial period.

Considerable scatter of the data shown on Chart 1 is, in great measure, attributable to biologic variability, but it may be mentioned that the conspicuous decrease of the pulmonary burdens at the 38th week was coincidental with a temporary discontinuation of exposure due to the Christmas holidays. The extent and severity of pulmonary pathology sustained by an animal during beryllium inhalation showed no correlation to its total lung load, but when the beryllium content of excised tumors was compared with that of surrounding nonmalignant pulmonary tissues, the former showed a distinct decrease on a per gram wet tissue basis (0.50 ± 0.35 versus 1.50 ± 0.55 µg of Be/gm). Evidently, this was mainly attributable to the dilution factor operating in the rapidly growing tumor tissue, although conceivably other factors, such as lack of continued local deposition due to impaired respiratory function and enhanced clearance due to high vascularity of the tumor, may also have played a role.

The pulmonary beryllium of these rats was tested with respect to its electrophoretic motility in a borate buffer of pH = 8.6 (0.030 M H3BO3 + 0.012 M NaOH). Migration occurred toward both the anode and the cathode in different specimens, suggesting association of the metal with one or the other of tissue protein fractions. Aldridge et al. (1) and Belman (2) have studied such beryllium proteinates in blood and in vitro, respectively; the immunologic aspects of human berylliosis (5-7, 27) also indicate the likelihood of the formation of such complexes (18). In some specimens, however, all beryllium accumulated at the cathode. This finding is not incompatible with the view that the beryllium in these cases was entirely ionic, but other evidence (21) indicates that the migration was, at least in part, due to surface charges on colloidal particles of beryllium hydroxide and orthophosphate formed through interaction of the inhaled ionic beryllium with tissue fluids (19, 22). The question of beryllium-protein

<table>
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<tr>
<td>Capacitance</td>
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<td>Powerstat position</td>
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<tr>
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<td>Developer</td>
<td>3.5 min E.K. D-19 1:4</td>
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<tr>
<td>Stop bath</td>
<td>0.5 min 1% CH3COOH</td>
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<td>Fixing</td>
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<td>Safelight and temperature</td>
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* Spec-Power, National Spectrographic Laboratories, Cleveland, Ohio; dual-grating emission spectrograph and spectrographic densitometer, Bausch & Lomb Inc., Rochester, N. Y.
association and the identification of the specific tissue proteins involved are subject to continued studies.

Beryllium in the tracheobronchial lymph nodes first reached detectable levels after 16 weeks of exposure (Chart 2). The subsequent course of accumulation appeared to be somewhat dissimilar to that observed in lungs: instead of coming to a plateau, these levels tended to show a maximum during the period from the 36th to the 52nd week of exposure, with subsequent decline while the animals were still in the chambers. Moreover, the difference in the accumulation rates between the sexes was found to be much more pronounced, and only partially attributable to the differences in body size. The average weight of a complete set of the six tracheobronchial lymph nodes was 0.09 gm for control males, 0.25 gm for exposed males, 0.13 gm for control females, and 0.20 gm for exposed females. It thus appears that males displayed higher efficiency than females in removing pulmonary beryllium through the regional lymph nodes, causing greater average enlargement of the nodes and also improved ability to resist the beryllium challenge; attritional mortality, as well as average body weight loss, was significantly lower in males than in females in this experiment (20).

Blood beryllium remained below detectable levels (<1-5 × 10^{-9} gm/ml) throughout the experiment. Acid and alkaline phosphatase activities of plasma were monitored and found to be unaffected by the inhalation exposure: the mean levels (in King-Armstrong units/ml ± S.D.) for acid phosphatase were 22.5 ± 5.5 in controls and 22.0 ± 3.0 in exposed; and for alkaline phosphatase, 41.5 ± 15.5 in controls and 39.5 ± 17.5 in exposed. Somewhat greater differences were found in the mean plasma alkaline phosphatase levels of exposed rats according to whether or not they had pulmonary cancer (36.0 ± 17.0 for tumor-free and 42.5 ± 20.5 for tumor-bearing animals).

**DISCUSSION**

The beryllium atom has amphoteric properties, resulting in appreciable hydrolysis of its common salts in aqueous solutions. This, in turn, causes these solutions to deviate from the neutral; the pH of the liquid disseminated in the experiment was 2.75. The neutralization of the inhaled aerosol is accomplished by the buffering action of pulmonary fluids, mainly through local precipitation of the hydroxides and phosphates (21, 22). The handling of inhaled beryllium sulfate by the mammalian lung thus becomes in some ways more similar to that of insoluble particulate matter than of substances which remain in dissolved form at neutrality. However, the solubilities of the precipitates which are formed are not altogether negligible; they are reported (16, 25) to be in the order of 10^{-9} moles/ml for the crystalline compounds, and they may be higher for colloids. It is thus possible to regard the freshly precipitated beryllium particles imbedded on bronchoalveolar surfaces as steady focal sources of Be^{++} and/or Be(OH)^{+} ions, continuously engaging and eventually overtaxing the buffering capacity of surrounding tissue fluids.

The fulminating and usually fatal acute chemical pneumonitis in animals exposed to high atmospheric concentrations (>0.1 mg/cu m) of soluble beryllium undoubtedly originates from this kind of primary injury, and the same mechanism may also be partially responsible for the chronic inflammatory response seen in animals at lower concentrations (30, 31). The question arises whether this acidic irritation may also be a factor in eliciting the carcinogenic response.

From the evidence now at hand this question would have to be answered negatively. In a study where the effects of equimolar amounts of inhaled beryllium sulfate and aluminum sulfate were compared (Vorwald and Reeves, unpublished data), the latter exposure was not productive of pulmonary cancer in the rat, even though acidity, hydrolysis, and the in vitro capability to exhaust tissue buffer reserves are very nearly identical for these 2 compounds and their potential to elicit a phagocytic response in the alveoli seems also comparable. However, the capacity to induce pulmonary cancer apparently depends on far subtler interactions with target cells, and in this respect beryllium appears unique among all light and most other metals.

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**Chart 2.** Tracheobronchial lymph node beryllium levels during and after BeSO₄ exposure.
The magnitude of pulmonary beryllium loads during inhalation is evidently governed by the competing processes of deposition and elimination, and the occurrence of a plateau appears significant because it suggests a tendency toward an equilibrium between these processes. The plateau phenomenon, and its quantitative but not linear dependence on the exposure level, was first observed by Reeves et al. (23), although Schepers (24) also reported the rapid attainment of an apparent saturation dose (8–10% of lung ash) of zinc manganese beryllium silicate in the lung of rats exposed to 26 mg/cu m of this dust for 9 months. With beryllium sulfate, however, Schepers found a perfectly linear increase for as long as 12 months during exposure, and in his experiments the increase of pulmonary beryllium levels continued at an even steeper rate after removal of the animals to clean air. This curious observation was interpreted as pulmonary redeposition of beryllium from extrapulmonary storage sites (24). No physiologic mechanism is known to us which would support this interpretation, and the observation itself was not confirmed in our studies.

According to more conventional views, the movement of inhaled beryllium between the lungs and extrapulmonary tissues is essentially unidirectional and depends primarily on whether the pulmonary beryllium is present in solution or as a particulate. Dissolved molecules or ions would be easily available for clearance through the blood stream and lymph channels by means of diffusion of the solutions through the bronchoalveolar tissue structures. The clearance of particulate matter as such would, on the other hand, depend on surface transport to the ciliary escalator, phagocytosis, and/or mural penetration of the dust into the interstitium and further into the lymph ducts and blood capillaries (3, 10, 11, 15).

In the interpretation of the plateau phenomenon, the possibility was considered that it might have resulted from a pulmonary beryllium clearance which proceeded entirely through the mechanism of limited but continuing solubility of precipitates formed in situ, and subsequent diffusion of dissolved beryllium out of the lungs. The pulmonary loads could then be assumed to depend, in a simplified model situation, on the elimination of a fixed percentage of the increasing load during, and of the decreasing load after, the inhalation exposure. Accordingly, the plateau would be explainable as the balance between a deposition-precipitation rate which keeps adding to the pulmonary load evenly and a dissolution-elimination rate which subtracts increasing amounts that are proportional to the loads existing at various times. Eventually, the dissolution-elimination rate would match the prevailing deposition-precipitation rate, resulting in the observed equilibrium.

Mathematical analysis of this hypothetical model allows calculation of the expected pulmonary load, \( y \), at any time, \( t \), by the differential equation

\[
\frac{dy}{dt} = s - ry
\]

where \( s \) is the amount of deposition and \( r \) the extent of clearance (as fraction of the load) of the inhaled material during a unit time period. This equation yields

\[
y(t) = \frac{s}{r} \left(1 - e^{-rt}\right)
\]

where the expression \(1 - e^{-rt}\) approaches unity with increasing \( t \), so that at very high values of \( t \)

\[
y(t \to \infty) = \frac{s}{r}
\]

or the quotient of the amount of deposition and the extent of clearance is defined as the plateau value. The difference between this plateau and the various preplateau loads \((s/r) - y\) accordingly becomes a direct logarithmic variable of \( t \). The solubility hypothesis of pulmonary beryllium clearance might therefore be tested on a plot of \( \log [(s/r) - y] \) against \( t \), because the slope of the resulting straight line depends exclusively on the value of \( r \), and if clearance is simply a consequence of the solubility of the deposited material in the lungs, then this value should be independent of sex.

Chart 3 represents such a plot for both males and females for the first 20 weeks of exposure. It should be emphasized that biologic variability as well as inadvertent inconstancy of certain experimental conditions such as atmospheric concentration and respiratory volume, as well as the night- and weekend-interrup-
tion of exposures, tend to make this kind of evaluation difficult, and our conclusions are at this time tentative. Nonetheless, it would seem that the straight lines which may be fitted to the points on Chart 3 are distinctly not parallel, showing that the rate of clearance of inhaled beryllium from the lungs during continued inhalation is not the same for both sexes. Accordingly, this evaluation shows that the solubility of the intrapulmonary precipitates should not be regarded as the only substantial factor in beryllium clearance; other mechanisms, which are contributed by the host and are apparently sex-dependent, are also involved.

The value of [(s/r) — y] (the ordinate on Chart 3) is in reciprocal relation to r, so that increasing clearance rates are manifested as diminishing degrees of steepness of the lines on this chart. The steeper slope of the line representing females is therefore interpretable as a less efficient pulmonary clearance in this sex. This agrees with the finding that accumulation of pulmonary beryllium in the tracheobronchial lymph nodes of females is much less than of males (Chart 2); it would therefore seem that it is the lymphatic clearance of pulmonary Be deposits which is less well functioning in female than in male rats. This could well account for the higher morbidity and mortality of females in this experiment (20).

The circumstances relating to pulmonary clearance may be further tested by analyzing the clearance curve after cessation of exposure. If the solubility of precipitates were the only controlling factor in the process of clearance, then this should follow the course of the common decay function

\[ y(t) = \frac{s}{r} e^{-rt} \]

and the value of y should decrease exponentially with increasing t. Accordingly, the plot of log y against t should give a straight line in animals sacrificed after a certain period of residence in clean air.

Chart 4 shows that this is clearly not the case in males (females are not shown due to insufficient number of samples), and the clearance curve appears as a composite of at least 2 phases; the first exhibiting a fast and the second a very slow rate. Less than half of the total pulmonary beryllium was cleared during the 4-week postexposure period, with the overwhelming amount of this clearance occurring during the 1st postexposure week.

This significant retardation of beryllium clearance after the rapid elimination of an initial amount may have several reasons. Among these, the following possibilities are immediately apparent: (a) the aging of the intrapulmonary precipitates with possible transition from a colloidal to a crystalline form, thereby substantially reducing the solubility rate; (b) the pathologic response surrounding some of the particles with scar tissue resulting from inflammation, and reducing the accessibility of the deposits to tissue fluids; and (c) formation of beryllium proteinates firmly embedded in the pulmonary architecture.

Accumulation of beryllium in the tracheobronchial lymph nodes following exposure of rats to beryllium sulfate was 1st shown by Stokinger et al. in 1950 (28). This accumulation evidently originates from that portion of the pulmonary load which entered the lymph channels, but the chemical form and mechanism of retention of beryllium in the tracheobronchial lymph nodes are at present not clear. One of the protein complexes suggested earlier (1, 2) may perhaps be implicated in this form; there is also evidence that soluble beryllium may exist as a complex with citrate (8). Amino acid complexes as well as adsorptive complexes in or on the surface of lymphocytes and/or of other cellular elements may provide further possibilities for the transport of beryllium to, and retention in, the regional lymph nodes.

The peaking of beryllium levels in the tracheobronchial lymph nodes, appearing coincidentally with the attainment of pulmonary plateaus, was not reported earlier. In view of the scarcity of existing knowledge concerning the physiologic behavior of lymph nodes under the influence of a toxic load, no certain choice is possible at this time between attributing this phenomenon to either diminishing accumulation or increasing dissemination of beryllium during the latter part of an inhalation exposure. The buildup of high lymph node loads would very likely favor an increased rate of systemic dissemination; but progressing pulmonary pathology during beryllium inhalation could also gradually impair the efficiency of normal clearance routes, and the amounts transferred to the lymph nodes could become less as exposure continued.

The ultimate site of accumulation of beryllium in the mammalian organism appears to be the skeleton (4, 29), although temporary deposits in the liver were also observed (1, 10). From the point of view of pulmonary carcinogenesis, however, that portion of inhaled beryllium is of greatest significance which is retained in the lungs for longer periods. The data presented in this paper suggest that this portion is in the range of one-half of the original pulmonary load, and it may be assumed that it has become incorporated into certain pulmonary cells. Firket (9), Reeves (18), Vorwald and Reeves (32), and, more recently, Gusek and Mestwerdt (12) showed by various methods, including histochemistry, ultracentrifugal fractionation, and electron microscopy, that the pulmonary beryllium content becomes localized in the cell nuclei. This localization may have significance in the elicitation of the carcinogenic response associated with beryllium inhalation, and in subsequent papers of this series we shall report on studies which may contribute to the understanding of the mechanism of this process in the experimental animal.
ACKNOWLEDGMENTS

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REFERENCES


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