Drugs Against Cancer: Stories of Discovery and the Quest for a Cure

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CHAPTER 13

The Bleomycin Story: an anticancer drug with a unique mode of action.

In 1957, Hamao Umezawa at the Institute for Microbial Chemistry in Tokyo was running a program to discover new anticancer drugs that microbes might produce. His group was testing samples for their ability to act against sarcoma tumors transplanted in mice. In 1962, they detected an active material produced by *Streptomyces verticillus* bacteria of the actinomyces family that had previously been a source of several antibiotics. The purified active material became the anticancer drug bleomycin, which actually is a mixture of chemical cousins with bleomycins A2 and B2 as the major components (Umezawa et al., 1967). Bleomycin became important in the cure of Hodgkins lymphoma and germ cell cancers of testis and ovary.

As of 1967, what was known about bleomycin was that it inhibited the growth of a sarcoma tumor in mice and inhibited the synthesis of DNA, but not RNA. It went to the lung and kidney, but not liver or brain. Chemically, it was water-soluble and had a tightly bound copper atom (Umezawa et al., 1967). The surprising significance of the bound copper atom only came to light years later. Throughout the next decade, it became evident that bleomycin could break DNA strands, but that some kind of chemical activation was required. But the data on the chemicals that activated bleomycin were very confusing and contradictory.

A breakthrough came at last around 1975, when Susan Horwitz and her colleagues at the Albert Einstein College of Medicine in The Bronx, New York discovered that as long as the copper atom remained bound to bleomycin, no DNA degradation occurred. But, when the copper atom was replaced by iron (ferrous ion), which could bind at the same bleomycin site as copper, then bleomycin became highly active in degrading DNA (Sausville et al., 1976). Another requirement was the presence of oxygen. The light then dawned as the so-

called Fenton reaction came to mind, a reaction in which ferrous ion reacts with hydrogen peroxide to generate highly reactive hydroxyl radicals that were known to break up DNA strands. Indeed, the Horwitz group showed that bleomycin, ferrous ion, and oxygen bound together in a complex (Burger et al., 1979). Moreover, the action of bleomycin on DNA was enhanced by the drug's ability to bind DNA by intercalation. Figure 13.1 shows bleomycin's chemical structure as Horwitz understood it in 1979. The structure was later modified slightly and confirmed by total synthesis as the reactive parts of the molecule became better defined (Figure13.2) (Hecht, 2000).

A unique feature of bleomycin's action on DNA was that it produced almost entirely double-strand breaks, rather than single-strand breaks, as shown by a beautiful experiment carried out in 1977 by Larry Povirk and his colleagues at Yale (Figure 13.3) (Povirk et al., 1977). Double-strand breaks are much more lethal than single-strand breaks, although the latter produce more mutations.

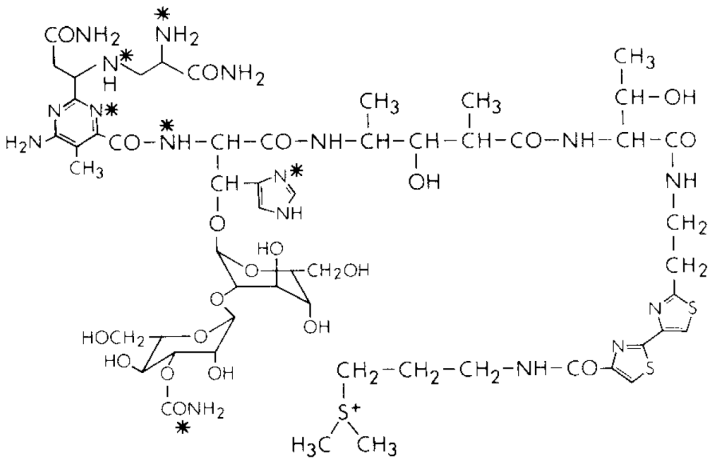

Figure13.1. Chemical structure of bleomycin A2 (later corrected slightly (Hecht, 2000)). Asterisks show possible metal binding sites proposed by Susan Horwitz and her coworkers (Burger et al., 1979).

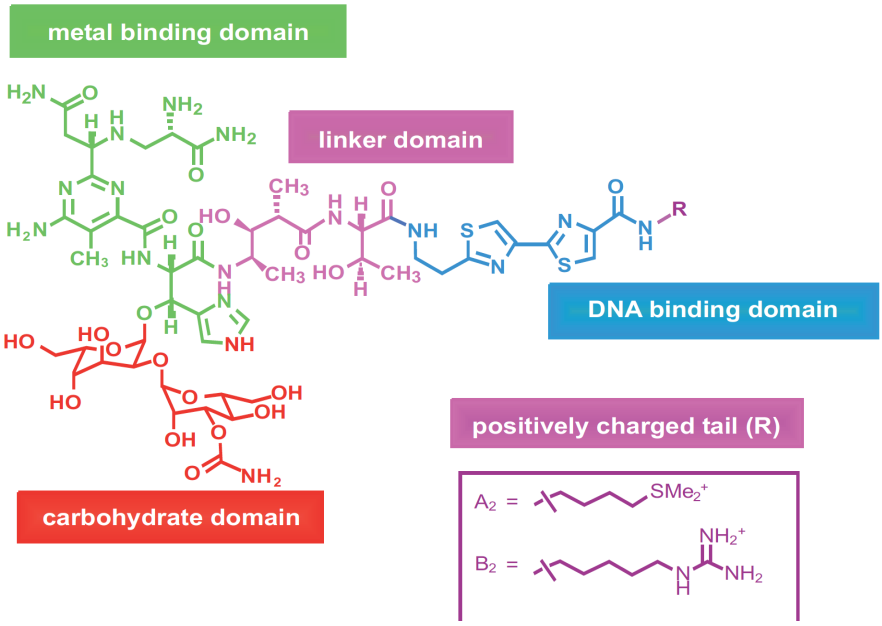

Figure 13.2. Structure of bleomycin, showing its functional parts (Yu et al., 2016). The positively charged tails that distinguish the 2 major forms of bleomycin are shown in the box at the lower right. These tails attach at the right end of the structure, where they are marked "R".

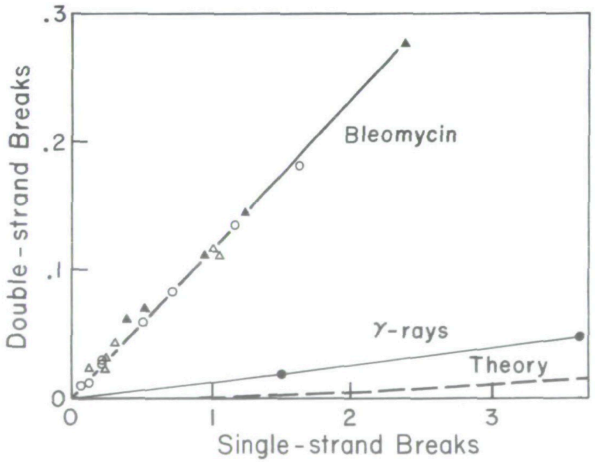

Figure 13.3. Bleomycin produced almost entirely double-strand breaks rather than single-strand breaks in DNA. The number of double strand breaks produced by bleomycin (upper curve) was far greater than the number that would have been expected from randomly close pairs of opposed single-strand breaks (curve labeled "Theory"). In these experiments, small double-stranded circular DNA was treated with bleomycin. A double-stand break would convert the circular DNA to linear DNA, whereas a single-strand break would not do so. The fraction of the DNA molecules linearized were measured and made for a highly sensitive and quantitative assay (vertical axis) (Povirk et al., 1977). x-rays on the other hand produced mainly single-strand breaks.

Effect of pH on the action of bleomycin.

My laboratory began studying bleomycin 1975, shortly after we had developed the alkaline filter elution method for quantitating DNA breaks in mammalian cells (Chapter 9). We didn't yet know about the role of ferrous iron – but that didn't bear directly on our studies of bleomycin-induced DNA damage in intact cells. The elution method was more sensitive and quantifiable than previous methods and allowed us to measure bleomycin's effects at low dosage, where cells began to be killed. Indeed, the extent of cell killing was precisely related to the amount of DNA strand breakage (Figure 13.4) (Iqbal et al., 1976; Kohn and Ewig, 1976). Looking back on those experiments of more than 40 years ago, it seems that they might have unexpected current clinical bearing on the toxic effect of bleomycin on the lung, as I will explain after relating the experimental problem we first had to solve.

We had to overcome two vexing problems that had given widely different results from one experiment to another. These inconsistencies had given Zafar Iqbal, a post-doctoral fellow in our lab who was carrying out those experiments, much anxiety and consternation. Looking over the data from the many experiments he had carried out, we noticed that experiments carried on Tuesdays seemed to give different results from those carried on Thursdays. It turned out that the different results seemed to depend in part on whether the experiments were carried out in glass as opposed to plastic tubes. Further tests showed that bleomycin indeed had a marked tendency to stick to some surfaces and not to others. If much of the bleomycin stuck to the tube surface, it would not be available to enter the cells. When we were careful to avoid the surface-sticking effect, much of the variability between experiments disappeared.

But not all of it! Some experiments still gave deviant results. Becoming aware of our consternation, our keen cell culture technician, Irene Clark, said that she thought the color of the medium in which the cells were growing was sometimes more yellowish at the time of the experiment than the usual red of fresh medium. The color indicated the pH of the medium. So … when we carried out all experiments in fresh, nicely red medium and avoided the surface-sticking effect, all variability vanished and the results became quantitatively beautiful (Figures 13.5 and 13.6) (Iqbal et al., 1976; Kohn and Ewig, 1976).

So, what about the color of the medium, you ask. Well, the color of the medium is due to a pH indicator added to help see the state or the culture: as cells grow, they make the medium more acidic, which changes the color from red to yellowish. Experiments carried out in yellowish medium would have been under more acidic (lower pH) conditions, suggesting that the toxic effect of bleomycin might depend on pH. That turned out indeed to be the case. Moreover, both cell killing and DNA damage were greater when cells were treated under mildly alkaline conditions (pH 7.5) than when treatment was under slightly acid conditions (pH 6.8) (Figures 13.5 and 13.6) (Kohn and Ewig, 1976).

But why is the action of bleomycin dependent on pH? One possibility that we considered was that bleomycin's histidine became positively charged at the lower pH, as would be

expected from the known chemistry of histidine. The additional charge could impair the ability of bleomycin to enter cells. Alternatively, it might impair its metal-binding activation.

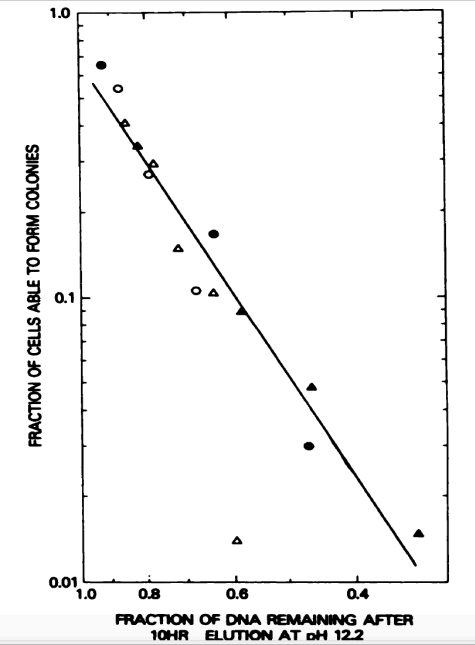

Figure 13.4. Killing of cells by bleomycin depended on the amount of DNA damage, which was higher when there was less DNA remaining on the filter. The cell killing (loss of the cells' ability to grow into colonies) had the same dependance on DNA damage regardless of pH or bleomycin concentration or duration of treatment. Mouse leukemia cells in suspension culture were exposed to bleomycin for various amount of time in medium buffered at pH 6.7 (open symbols) or pH 7.5 (filled symbols) and bleomycin concentrations of 13 (triangles) or 50 μg/ml (circles) (Kohn and Ewig, 1976).

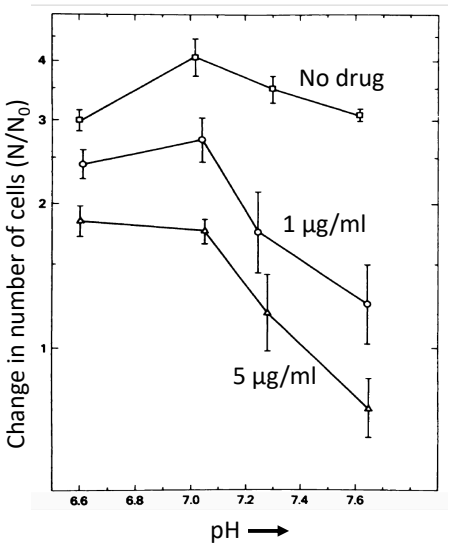

Figure 13.5. At pH above 7.0, bleomycin became increasingly effective in killing cells. The graph shows the change in number of mouse leukemia cells after 20 hours of incubation in suspension culture at the indicated pH containing the indicated concentration of bleomycin (Kohn and Ewig, 1976).

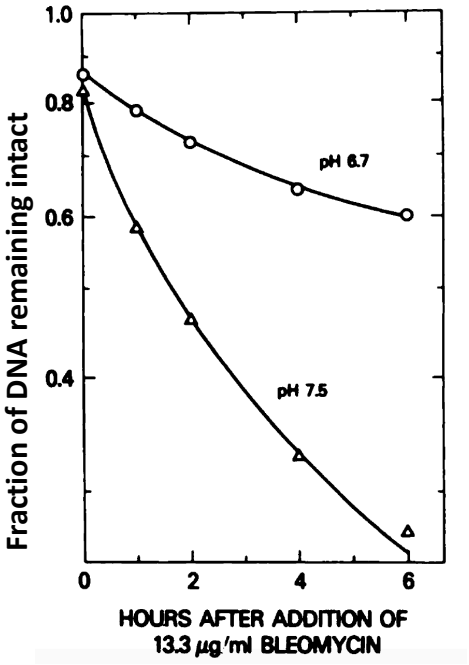

Figure 13.6. Bleomycin degraded the cells' DNA faster at pH7.5 (lower curve) than at pH6.7 (upper curve). Cells were treated in growth medium at the indicated pH. The vertical axis shows the fraction of the DNA remaining free of strand breaks at various times after addition of bleomycin to the medium (Kohn and Ewig, 1976).

Bleomycin in cancer treatment.

Choice of drug combinations for cancer chemotherapy aimed to match drugs whose toxicities affected different organs or tissues. Bleomycin was often chosen because it was not very toxic to the bone marrow or heart. Its toxicity was instead mainly to the lung. That led to bleomycin becoming an effective part of the treatment of Hodgkin lymphoma and of germ cell cancers of the testis and ovary.

Hodgkin lymphoma accounted for about 10% of lymphomas and about 1-2% of all cancers (Cuceu et al., 2018). However, it often occurred in younger people, which made it important to have treatments that allow long-term survival. Bleomycin had an important role alone the road to effective treatment that eventually cured about 80% of patients, although its contribution was limited by its unusual toxicity to the lungs. Patients who at first appeared to be cured had a shorter survival time than would have been expected. Also, about 20% of patients did not respond to the drugs. The treatments had dramatic successes but failures as well.

In his book "The Death of Cancer", Vincent DeVita tells the remarkable story of how Hodgkin lymphoma (originally called Hodgkin's disease), was cured by a combination of 4 anticancer drugs . Hodgkin lymphoma is a malignant tumor composed of particular kinds of lymphatic cells. It is a special kind of lymphoma. DeVita and his colleagues at the National Cancer Institute had chosen to attack this cancer because of its known growth characteristics that indicated that most or all of its malignant cells would be in the cell division cycle and therefore vulnerable to chemotherapy drugs. Moreover, Hodgkin lymphoma tumors had been seen to shrink in some patients treated by nitrogen mustard (nitrogen mustard is a topic in Chapter 1). Therefore, nitrogen mustard was one of the drugs DeVita and his colleagues chose in their 4-drug combination.

They thought that a combination of 4 drugs would be needed to clear out all of the malignant cells, based Howard Skipper's quantitative findings in leukemia in mice. They combined drugs that had different modes of action and different toxicities, so as to attack the tumor from different angles and minimizing the net toxicity. They called their combination, MOPP, for nitrogen mustard (M for mustard), vincristine (O for its other name, oncovine, see Chapter 12), procarbazine (another kind of alkylating agent, see Chapter 2) and prednisone. Each of these drugs were known to kill or inhibit malignant white blood cells and had a different mechanism of action.

Their attack on Hodgkin lymphoma was similar to the 4-drugs combination, VAMP, that had successfully cured childhood leukemia. That story was told by John Laszlo in his book "The Cure of Childhood Leukemia". However, there were two crucial differences that had to be considered when it came to choosing a drug combination to combat Hodgkin lymphoma.

First, leukemia cells populate the bone marrow, and to get cures the bone marrow had be cleared out of all cells. Hodgkin lymphoma, on the other hand, consists of solid tumors that

usually do not harm the bone marrow. Therefore, contrary to drugs for leukemia, drug choice for Hodgkin lymphoma did not try to eliminate the blood cells in the bone marrow.

Second, although most leukemia cells undergo cell division within a couple of weeks, the malignant cells in Hodgkin lymphoma often wait for a longer time before they divide. Therefore, the drugs that kill the malignant cells during their cell division period had to be administered to patients over a longer period of time than was the case with leukemia.

The 4-drugs combination, MOPP, had been successful in treatment of Hodgkin lymphoma for several years, when bleomycin came along, featuring low bone marrow toxicity. In 1975, Gianni Bonadonna at the Instituto Nationale Tumori in Milan, Italy used bleomycin to replace the DNA-damaging nitrogen mustard in new drug combinations. The combination of bleomycin, doxorubicin, vinblastine, and dacarbazine (ABVD), was eventually found to be at least as effective as MOPP or its variants, and became the standard treatment for Hodgkin lymphoma (Canellos et al., 2014).

Initially, the Bonadonna group continued to use the MOPP combination, but added the new bleomycin and doxorubicin-containing combination (ABVD). Their new treatment of Hodgkin lymphoma alternated MOPP and ABVD. MOPP alone cured about 45% of the patients, whereas the MOPP/ABVD alternating treatment increased the cure rate to about 73% (Bonadonna et al., 1986) (Figure 13.7). It was later found that treatment with ABVD by itself was at least as effective, and gave less toxicity (Canellos et al., 2014).

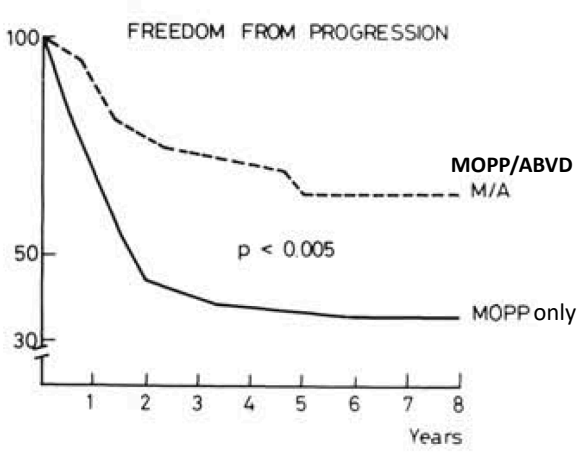

Figure 13.7. Survival of patients with advanced Hodgkin lymphoma was increased by adding a bleomycin- and doxorubicin-containing drug combination (ABVD) to the MOPP treatment (dashed curve). The curves show the fraction of patients free of progression or relapse of the tumor as a function of the number of years after treatment. Once patients had survived for 5 years, they were cured: the curves flattened out after 5 years, and there was no further progression of the tumor for at least up to 8 years. Patients who were treated with the then standard treatment with the MOPP drug combination (lower curve) had a 45% chance to cure. However, when the bleomycin-doxorubin-vinblastine-dacarbazine combination (ABVD) was added in alternation with MOPP, the cure rate increased to 73% (upper curve) (Bonadonna et al., 1986). Later, it was found that ABVD by itself was as effective and less toxic than the alternating MOPP/ABVD treatments.

Hodgkin lymphoma contains unusual giant cells, called Reed-Sternberg cells (Figure 13.8). These cells are giants, much larger than other cells in the tumor; they often have more than one nucleus and the nuclei have prominent nucleoli where ribosomal RNA is made. Although there are relatively few of those cells in the tumor, they seemed to be the malignant cells that divided to produce the other cells that make up the bulk of the tumor. It became possible to identify those truly malignant cells in Hodgkin lymphoma tissues by means of antibodies that bind to specific “marker” proteins, called CD15 and CD30, on the surface of those cells (Cuceu et al., 2018). The Reed-Sternberg cells seemed to arise by cell fusion from B-lymphocytes. Their chromosomes were particularly unstable, in that the number of chromosomes in each cell tended to vary and there often were recombinations between different chromosomes.

Most of the cells in a Hodgkin lymphoma tumor were killed by anticancer drugs. But the Reed-Sternberg cells in the tumor were harder to kill and grew new tumors after drugs had shrunken much of the tumor bulk. Thus, the tumors would seem to have been almost eliminated, but would soon grow again. The treatment therefore had to be strong enough (despite greater toxicity) to eliminate those truly malignant cells, which are like “stem cells” that gave rise to the bulk of the tumors.

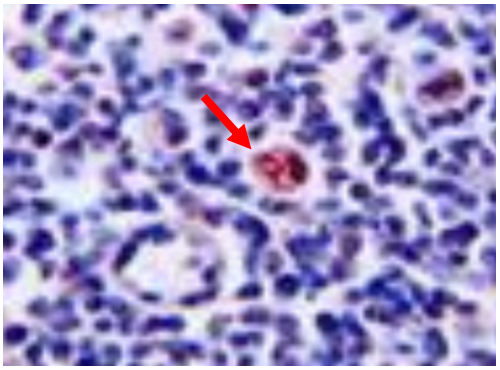

Figure 13.8. A Reed-Sternberg cell in a Hodgkin lymphoma tumor (arrow) (from (Cuceu et al., 2018) *(permission needed to use their figure 1B)*. The cell looks pink, because of staining for the Li-Fraumeni protein, p53, which is often prominent in these cells.

Bleomycin contributed also to the effective treatment of germ cell cancers of testis and ovaries as well as of teratomas that occur usually in those organs and of the formerly deadly choriocarcinomas that form in the placenta during pregnancy. Germ cells in humans are the sex cells that develop into sperm and ova.

In 1974, a group at Indiana University cured advanced testicular cancer in 57% of patients with bleomyin in combination with cisplatin and vinblastine. Investigators eventually found an optimum dosage and treatment schedule that cured nearly all of the patients (Einhorn, 1981). Although rare, testicular cancer was the most common cancer in the 15 to 35 age group, and it was one of the first cancers at an advanced stage that could usually be cured by chemotherapy. (Choriocarcinoma was the first cancer to be cured by chemotherapy (Chapter 5)).

Lung toxicity

The bleomycin-containing ABVD drug combination cured 80% of Hodgkin lymphoma patients. A major problem however was bleomycin-induced lung toxicity, which was an inflammation of the bronchial tree that sometimes led to irreversible fibrosis and permanently reduced lung function (Figures 13.9 and 13.10). The huge amount of fibrosis produced in the lung by bleomycin can be appreciated by comparing with normal lung in Figure 13.11. The lung toxicity of bleomycin was of particular concern, because many of the cured patients were relatively young and could have breathing problems for the rest of their lives. Despite much effort to find ways to counter bleomycin's lung toxicity, the most reliable way to avoid the toxicity was to reduce the dose of the drug, which however reduced its therapeutic effect. Early detection of bleomycin's lung toxicity was important, so that the dose could be reduced promptly. But the lung inflammation caused by bleomycin was difficult to distinguish from a bacterial lung infection. An early clinical trial

already indicated that, if bleomycin lung toxicity could be avoided, 90% of Hodgkin lymphoma patients treated with ABVD would be cured (Figure 13.12) (Martin et al., 2005).

The main lung abnormalities caused by bleomycin was damage to the cells of the inner linings of the bronchi, alveoli, and blood vessels (Sleijfer, 2001). These cells would be at the interface of the transfer of carbon dioxide to the expired air, and consequently might undergo a pH change that could make the cells more sensitive to bleomycin, as suggested above. Since bleomycin is activated by oxygen, the freshly oxygenated parts of the lung may be particularly sensitive to damage by the drug.

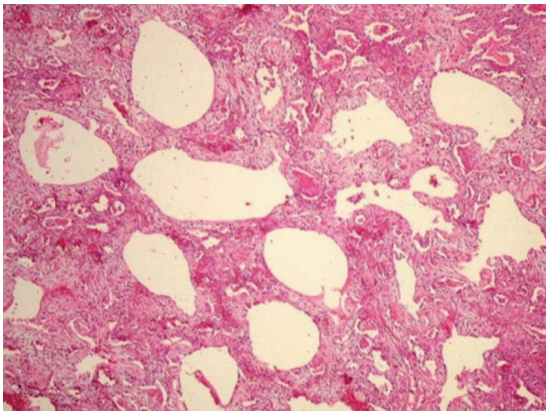

Figure 13.9. Lung biopsy tissue sample from the lung of a patient with bleomycin-induced toxicity, showing large amounts of inflammatory cells and fibrosis between the alveoli that greatly impaired the alveolar air spaces from opening (Reinert et al., 2013) *(permission need to use their figure 1)*.

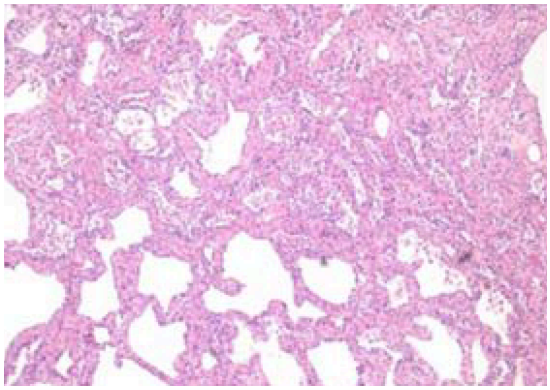

Figure 13.10. Lung tissue of a 20-year-old man who was cured of metastatic testicular cancer by a combination of bleomycin, etoposide, and cisplatin. However, he developed severe lung toxicity that required transplantation of both of his lungs, which was successful. The walls of the alveoli of the removed lung are thickened or completely overgrown by inflammatory cells, especially in the upper tight of the slide. The overgrown cells and fibrosis prevented opening of most of the air spaces of the alveoli. This was the first reported case of lung transplantation for bleomycin toxicity (Narayan et al., 2017) *(permission need to use their figure 2B)*. (*Permission needed.)*

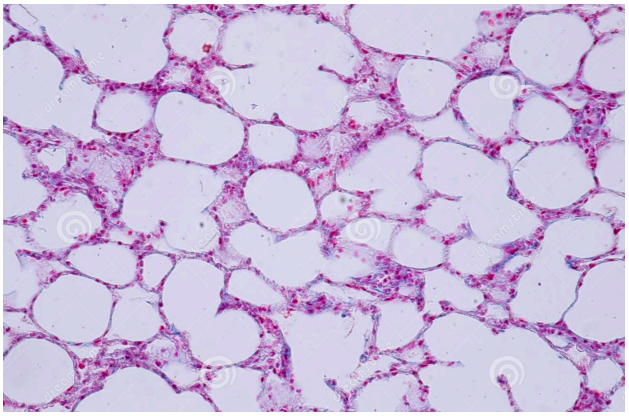

Figure 13.11. Normal lung for comparison with the previous two Figures. Note the thin epithelial linings of the air-filled alveoli. *(Freely available from Wikipedia.)*

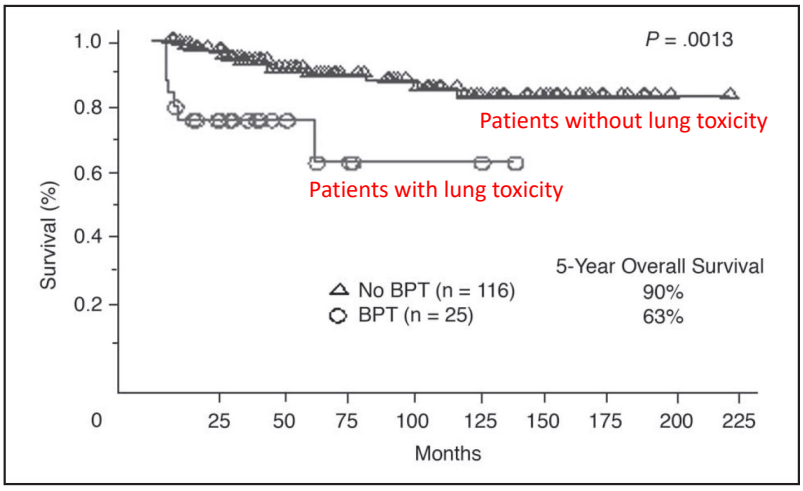

Figure 13.12. Survival of Hodgkin lymphoma patients treated with a bleomycin-containing combination (usually ABVD or MOPP/ABVD). The graph shows the percent of patients surviving versus the number of months after treatment. After 5 years, 90% of patients who did not have lung toxicity were still alive (upper curve), whereas only 63% of patients who did have lung toxicity survived (lower curve) (Martin et al., 2005).

If its lung toxicity could be controlled, bleomycin could help cure many more cancer patients. Many attempts were made to control the lung toxicity of bleomycin. The receptor kinase inhibitor, nintedanib, reduced bleomycin-induced lung damage in mice. The fibroblast inhibitor, pirfenidone, reduced bleomycin-induced in the lung of rats. But how effective these small-molecule drugs were in bleomycin-treated patients was uncertain. The large cyclic peptide, everolimus, inhibits the proliferation of fibroblasts, suggesting that it might inhibit fibrosis in the lungs of bleomycin-treated patients. Clinical trials of this

drugs however were disappointing. Drugs that tended to keep the bronchi open were also ineffective (Della Latta et al., 2015).

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