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Toxicity of Particles: A Brief History

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Over the last few decades, a certain number of pathologies have been directly linked to various kinds of inorganic dust affecting subjects exposed to these substances in the workplace. As a consequence, public health authorities have become increasingly interested in determining the effects particles can have on health. But analytical investigations of pollution, along with other evidence, has shown that exposure to dusts is not limited to workers in specific sectors. In fact, it may also affect the population at large, a finding that has led to the setting up of think tanks, closer assessment of different types of pollution, and research specifically devoted to the toxicology of dusts. Note also that the terminology itself has evolved. In particular, the word 'dust' has gradually been replaced by 'particulate matter', although both terms refer to the solid fraction in aerosols.

The aim of the present chapter is to summarise the context and the facts that have led to the area of investigation we now call particulate toxicology. We begin with the sociological and technical features that have made the study of toxicity what it is today. For chronological reasons, we then define the pathologies caused by exposure to inorganic dusts, before going on to describe the particles responsible for pathogenic effects. To explain the mechanisms by which particles can act, we discuss methods for investigating toxicity and the ways they have evolved. On this basis, hypotheses are formulated about the mechanisms leading to observed biological effects. Before concluding, we shall consider the results of toxicological studies carried out up to now to assess the toxicity of particles occurring in professional and general environments, or likely to be generated in such environments, but also some questions which have not yet been answered, or which have arisen from earlier experiments.

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1.1 Sociological and Technical Factors Conditioning the Study of Particle Toxicity

Concern over the toxicology of inorganic dusts was first inspired by the discovery of pulmonary pathologies in workers with occupational exposure to such substances, e.g., in mines [1, 2]. In an interesting chapter on pneumoconioses in mines in the north of France, Amoudru summarises the steps leading to the recognition of these illnesses as work-related [3]. Note that it took over a hundred years to fully recognise this fact. Indeed, while lung diseases had been observed in several European countries, including France, at the beginning of the nineteenth century, it was not until 1945 that a statute was published recognising silicosis as a work-related disease. There were several reasons for this delay: medical controversy which delayed the undeniable recognition of the risk, factors leading to confusion, e.g., silicosis favours infectious pathologies like tuberculosis, and world political events which postponed application of the decision to recognise silicosis as a work-related disease until after the war. In the case of asbestos, purely economic motives of industrial protectionism hampered progress on the research front [4, 5].

Up to now, the toxicology of inorganic dusts has mainly been concerned with respiratory problems, because the principal route by which particles could enter the organism was of course inhalation. Once the particles causing pathological effects had been identified, experiments could be devised to determine the consequences of inhalation in tissues and cells, in order to understand the relevant mechanisms and determine the factors involved in the cell's response. In parallel, other research was investigating the physical, chemical, and physicochemical properties of the particles that led to this biological activity.

The relationship between exposure and pulmonary pathology in humans were identified by the end of World War II, but the experimental work was only published around the end of the 1960s. Returning to the example of dust in the coal mines, experimental data only became available in the 1950s. It is interesting to note that, in his autobiography, Dulbecco mentions that at one point, somewhere around 1946, he wanted to investigate certain illnesses caused by dust inhalation which it seemed concerned mainly miners. But the results of morphological analyses based on lung sections were insufficient to explain the causes of the lesions he observed, and unfortunately this eminent scientist did not pursue his efforts in this field [6]. A particle toxicology journal presents a list of European research programmes launched under the auspices of the *Communauté européenne du charbon et de l'acier* (CECA),[1] founded in 1951, to evaluate the effects of carbon and silica dusts. Note that the first dates back to 1955, and refers to medical issues [7].

The significant technological developments of the nineteenth and twentieth centuries, fuelled by the requirements of war, led to several groups of workers

[1] European Carbon and Steel Community.

being exposed to pollutants. Asbestos provides a second example of a harmful agent for certain groups of workers, and which further stimulated research into particle toxicology. The considerable increase in the use of asbestos fibres during World War II and the emergence of the associated afflictions only instigated research after a delay of about 20 years. The first consequences to be recorded were of pulmonary asbestosis. The role played by asbestos in the development of this pulmonary disease was described as early as the 1920s by several authors who had observed asbestos workers [8, 9]. Regarding the relationship with lung cancer, the inaugural study is often taken to be the work by Lynch and Smith [9, 10]. An increase in the incidence of lung cancer in the case of pulmonary asbestosis was subsequently reported by Doll in 1955, and then a few years later, an article was published about the high level of mesothelioma affecting workers in asbestos mines in South Africa, as well as other inhabitants of the region [11, 12]. The impact of this paper was twofold, because it identified the harmful effects of asbestos, but it also recognised this cancer as a primitive pleural tumour. Indeed, the reality of these tumours was debated [13].

With the case of asbestos, toxicology acquired a new feature. Whereas for carbon and silica, it was mainly in the production sector that the pathological consequences of exposure were being felt, in the case of asbestos, similar pathologies were being observed in a second sector, namely, people using asbestos-based materials (several trades, notably the building trade). This new wave of asbestos-related illnesses, sometimes called the second wave, led some to raise the question of a possible third wave, one which might reach people exposed for non-professional reasons in a general environment, e.g., buildings containing asbestos, natural pollution in regions with outcrops of asbestos-containing rocks, etc. [14, 15].

The evolution in the type of population affected by the harmful consequences of dusts, going from production to applications and the general environment, continues today with regard to the populations exposed for non-professional reasons. Indeed, we are now concerned about exposure to dusts of human origins, viz., fine particles (FP) and ultrafine particles (UFP). Examples of sudden increases in mortality associated with pollution peaks have raised concern in this area [16–18]. In parallel with the greater number of different populations affected by respiratory problems, other pathologies related to exposure to particles have had to be taken into consideration, such as cardiovascular disease [19]. With the emergence of nanoparticles (NP) resulting from nanotechnological developments, this diversification in the nature and origin of the particles on the one hand, and in the consequences for the health on the other, also affects sectors that are not directly in contact. In the case of NPs, apart from the production sector, applications, and the general environment, an ecological dimension has also come into being [20]. Concerning pathologies, the respiratory and cardiovascular systems are no longer the only ones to be affected, since the question of toxicity is now raised with regard to other sites, like the nasopharynx, brain, and kidneys [21].

Another important point must be taken into account, related to the technology, when considering the long term consequences of particle toxicology. In the 1950s, the relevant journals were mainly medical. But from the 1970s to 1980s, the possibilities for publication increased significantly, facilitating the exchange of information between different groups involved in this line of research. Furthermore, international exchanges between the various research centers became commonplace with the proliferation of conferences and seminars. Availability of information through numerous publications meant that results of studies and inquiries in the workplace became known to research teams who could then develop toxicological studies on animals, and subsequently, in vitro approaches on cell cultures. The fact that independent groups were now involved was a key factor in the development of this research. By comparing results on the international level, hypotheses could be formulated regarding the mechanisms involved, while warnings could be sent out regarding the potential toxicity of new products, and in some cases, help could be provided to formulate regulatory measures. The improved means of communication was an important factor in the evolution of research in particle toxicology. It should be borne in mind that Internet is a recent source of exchange between research scientists. Although this network was established by the end of the 1980s, email and file transfer protocols were only available to a few at the beginning, and it was only around 1995 that they became widely accessible [22].

1.2 Pathologies Caused by Inorganic Dusts

1.2.1 Pneumoconiosis

Pneumoconiosis is a pathology due to the presence of exogenous particles. These particles accumulate in the lungs, causing a tissue reaction associating cell inflammation and macrophages. Together these can form so-called foreign-body granulomas, containing giant multinuclear cells. Carbon dusts cause various lung diseases, including fibrosis, emphysema, chronic bronchitis, and an alteration of lung function [2, 23, 24]. Exposure may be associated with crystalline silica, depending on how the carbon is extracted.

Silicosis is a common form of pneumoconiosis caused by the deposition of silica particles in the lungs. Apart from activities associated with coal mining, it is encountered across a broad range of occupations related to metal mining and quarrying, the building industry, etc. [25]. On a histological level, it is a nodular fibrosis formed by fibrohyaline tissue [26]. This respiratory disease is still observed today, despite the decline in coal mining [27].

Asbestosis is caused by exposure to asbestos, and occurs in the form of a diffuse interstitial fibrosis [28]. In countries where the use of asbestos has been forbidden, it has become much less common, because it is a pathology that generally develops only after exposure to very high doses. Pleural plaques

are benign lesions caused by asbestos. This fibrosis is usually localised in the parietal pleura and evolves by calcifying [28].

Forms of pneumoconiosis induced by exposure to other types of dust have also been identified. These are mainly due to accumulation of dusts, some of which may evolve into fibrosis. They are caused by metal compounds of iron, aluminium, tin, or barium, but also by beryllium, and are manifested in the form of granuloma or fibrosis, e.g., siderosis, berylliosis, aluminosis, etc. [29].

Fibrosis results from increased synthesis of fibrous tissue, collagen, and proteins of the conjunctive tissue. It occurs in the pulmonary parenchyma, around the bronchi and in the pleura, and is associated with a tissue repair process. The increased amount of collagen and fibronectin come from a greater synthesis of proteins by fibroblasts in the extracellular matrix and/or an increase in the number of these cells. This in turn occurs in response to tissue lesions and factors emitted during inflammation by macrophages and neutrophils, both in the lungs and in the pleura [30, 31]. Studies carried out so far to investigate the fibrosing effects of particles have been based on this fibrogenesis mechanism [24]. Recent work discusses the role of inflammation in these tissue repair processes, attributing a role to the pneumocytes of the respiratory epithelium in the initiation and development of this process of pulmonary fibrogenesis [32].

1.2.2 Cancer

Cancer results from proliferation of cells exhibiting genetic alterations acquired over successive divisions. This neoplastic transformation involves several stages. During the process, various modifications occur in the cell's genetic material (mutations, deletions, translocations, etc.) and in the regulation of functions (control over DNA integrity, proliferation, recycling, and apoptosis). These modifications have several consequences in their turn:

- on gene expression, which is deregulated both qualitatively and quantitatively, and in terms of the expression rate;
- on the equilibrium between cell proliferation and mortality;
- on the relationship between the cell and the extracellular environment.

On a molecular level, the neoplastic transformation has been described as a mechanism of oncogene activation together with the silencing of tumour suppressor genes, and this model has been validated by observations and experimental studies [33]. Other studies have led to the proposal of a more general mechanism underlying the neoplastic process, this time involving several genes [34]. Hanahan and Weinberg have suggested that cells should acquire six hallmarks indicating their neoplastic character, viz., growth autonomy, resistance to antiproliferation signals, resistance to apoptosis, the potential for unlimited replication, sustained angiogenesis, invasion, and metastasis [35]. Recently, a seventh indicator of neoplastic transformation has been put forward, viz., inflammatory conditions [36]. The evolution of the cell during tumor growth

is sustained by chromosomal instability. This generates an alteration of the genetic material which is transmitted to the daughter cells during successive divisions [37,38]. The chromosomal aberrations found in tumours are indicators of genetic deregulation and chromosomal instability.

Neoplastic evolution is accompanied by phenotypic modifications, such as loss of contact inhibition between cells, independence from growth factors for proliferation, abnormal karyotype, genetic disequilibrium, and differential gene expression as compared with the normal cell. Toxicological tests are based on these modifications in the field of oncogenesis. They use techniques for revealing one or more phenotypic changes associated with this neoplastic evolution.

1.3 Particles Causing Pathogenic Effects in the Airways and Respiratory System

Particle toxicity is often discussed in cases where exposure is by inhalation in the professional environment, because the associated pathologies are generally discovered in the workplace. But exposure may happen in the environment as a whole, and not just in the vicinity of the sources of contamination, but some distance away, transported by atmospheric currents.

1.3.1 Origin of Particles

The origin of particles that have undergone toxicological studies has already been mentioned. These are particles generated by human industrial activities, industrial applications, or products used in everyday life. Stocks of samples have sometimes been established to be distributed to different research groups for the purposes of toxicological studies. This was the case for silica, by setting up stocks from different mines, particularly in Germany, and also for asbestos, through the action of the *Union internationale contre le cancer* (UICC), which prepared samples of each kind of fibre [39,40]. More recently, samples of artificial inorganic fibres have been distributed for various studies. These were glass, rock, slag, and refractory ceramic fibres [41]. Regarding fine and ultrafine particles (or nanoparticles), such as PM_{10} and $PM_{2.5}$, carbon black or titanium oxide and carbon nanotubes, this has not been done systematically. Although certain sources have been favoured for these particles, for others, they turn out to be very varied. It should be borne in mind that, in the general case, the samples used for experimentation are not always fully representative of the particles to which subjects have been exposed, owing to the great diversity of possible sources, whether they be natural or synthetic. Moreover, it would be difficult to test this diversity, due to lack of information regarding the nature of the exposure and the wide range of different particles.

1.3.2 Types of Particle

Carbon Dusts

These are complex compounds whose composition depends on the mine. These carbon-containing dusts contain various silicates, carbonates, and sulfates.

Silica

Silica is composed of silicon dioxide (SiO_2). There are several types, with the same chemical composition, but with different crystal structure and cytotoxic activity, viz., tridymite, crystobalite, and quartz. There is also a non-crystalline form, viz., amorphous silica, opal.

Asbestos

There are several types of asbestos. The term covers fibrous silicates in hydrated crystalline form, with various possible cation compositions, e.g., Mg, Ca, Na, Fe [42]. There are several forms of asbestos, with different structures and chemistry, mainly used in industry: band silicates such as the amphiboles, e.g., crocidolite, amosite, and anthophyllite, among others, which contain different cations Mg, Fe et Na, and sheet silicates such as phyllosilicate, and chrysotile, which is a hydrated magnesium silicate [43]. The sheets are rolled up around a central axis, giving the elementary fibril a multilayer, hollow tube structure. Today, asbestos is by far the most widely studied type of particle.

Other Fibres

According to the definition provided by the World Health Organisation (WHO), a fibre is a solid particle, either natural or artificial, with an elongated shape and parallel edges, with length greater than 5 μm and aspect ratio (length to diameter) greater than 3.

Among the other natural inorganic fibres, the main one to attract the attention of toxicological studies has been erionite, a zeolite mineral, which is basically aluminium silicate containing Na, K, and Ca. This is due to epidemiological observations associating mesothelioma with environmental exposure to these fibres [44]. In addition, many kinds of synthetic inorganic fibre such as rock wool, slag wool, and glass wool, or again refractory ceramic fibres, have been studied owing to their broad spectrum of applications, including their use as an asbestos substitute. These are silicates with different aluminium contents, and also different alkali metal and alkaline earth cations. Their chemical composition is very varied, depending on the application [45]. Research in this field has led to a notion of biopersistence, to be defined and discussed later.

For the record, one should also mention that synthetic organic fibres have been studied, e.g., para-aramid and aramid, used as strengthening materials

[25]. Recently, carbon nanotubes have attracted some attention, owing to the similarity of their physical characteristics with those of asbestos, which raises a doubt over their potential toxicity [46].

Fine and Ultrafine Particles. Nanoparticles

FPs and UFPs began to attact attention with the problems arising over pollution peaks. These terms cover a wide range of particles, from chimney smoke to carbon black and titanium oxide, not to mention particles contained in the surrounding air. Their chemical composition and structure are thus highly diverse. More recently, the development of nanotechnology has witnessed an expansion of the world of nanoparticles, which have applications across a very broad range of situations.

Studies of atmospheric pollution require the collection of particles according to their aerodynamic characteristics. Sampled particles are separated by reference to their aerodynamic diameters (AD): PM_{10} and $PM_{2.5}$ (AD less than 10 μm and 2.5 μm, respectively). Ultrafine toxicity studies suggest that effects were probably related to the ultrafine fraction, leading to the hypothesis that these UFPs are potentially more toxic than the FPs [47].

The development of nanotechnology created further sources of UFPs, with the synthesis of nanomaterials or the use of UFPs in a wide range of products. Nanoparticles are particles with at least one dimension of nanometric size, so they can be included with the UFPs. However, at the present time, the term UFP is generally reserved for naturally occurring particles, or man-made particles that have been unintentionally produced, while NP is used for particles produced by or resulting from the field of nanotechnology. Recent AFSSET reports in France survey the current situation with regard to toxicity and health risks raised by nanomaterials [48, 49]. According to one of these reports, nanomaterials are classified into four families depending on the form in which they are used [48]:

- in dispersed form, either random or ordered,
- in the form of nanowires or nanotubes,
- in the form of a thin film,
- in compact form.

The small size of NPs bestows special physicochemical properties upon them, which can make them highly reactive in a biological context. Nanoparticles resulting from nanotechnological activities can thus be produced as such or result from the manipulation of larger samples of material, e.g., by milling, degradation, etc. As far as chemical composition is concerned, nanoparticles may be metals, metal oxides, polymers, composite materials, or even biomolecules. Although of nanometric size, these particles tend to agglomerate and form aggregates, thereby increasing their overall size.

1.4 Evolution in the Methods for Investigating Toxicity

The methods for investigating particle toxicity have changed enormously since the first studies to be found in the literature, which date roughly from the middle of the twentieth century. Although earlier work is mentioned in several papers, it was only from the 1950s that a continuous and coherent literature came into being with the studies on silica, and later asbestos.

Subsequent work aimed not only to determine physiopathological effects, but also to understand the mechanisms of particle action. This was achieved through anatomopathological studies which, associated with other techniques like immunohistochemistry and histochemistry, then the main methods for characterising lesions, were able to identify the proteins involved in these mechanisms. Specific effects were observed in certain species (alveolar lipoproteinosis) [50]. Morphological observations led to in vitro studies on growing or surviving cells, and in particular on alveolar macrophages and fibroblasts. On these cells, the particles having exhibited cytotoxicity, revealed by cell viability assays, associated with the internalisation of the particles by the cells, work was then carried out to determine the nature of the interactions between particle and cell. Since the viability assays were based on examination of alterations in membrane integrity, 'model' cells were used. These were red blood cells with no capacity to internalise the particles, but providing information about the interactions between the cytoplasmic membranes and the particles. As data accumulated, efforts were made to understand the physiopathological mechanisms, reaction to foreign bodies, inflammation, and fibrosis, by determining the responses of specialised cells, macrophages, and fibroblasts. Then, with confirmation of the connection between cancer and exposure to certain types of particle, others investigated the effects on and responses of epithelial cells. It should also be noted that these methods were greatly speeded up by the commercialisation of cell culture equipment such as cell culture flasks, throwaways, and industrially produced culture medium.

1.4.1 Studies on Animals

To begin with, the means available for studying particle toxicity consisted in exposing animals, usually rats, but to some extent also guinea pigs and mice, in inhalation chambers. Intratracheal instillation was also used early on [51, 52]. This methodology brought in the possibility of anatomopathological studies which, depending on the exposure time, evaluated the fibrosing or oncogenic potential of the particles. Exposure by inhalation is a method requiring a large amount of costly equipment, e.g., systems for aerosolisation of the particles, dedicated inhalation chambers, large quantities of the particle, etc. It is assumed to reproduce a type of exposure that can be taken as realistic as far as human exposure is concerned. However, exposure doses are sometimes difficult to specify, e.g., dusts from animal fur. Another commonly

used method is intratracheal injection of particles in suspension in a physiological solution. This technique is not physiological, but the exposure dose is then clearly determined. On the other hand, there are various disadvantages, because the dose may be poorly distributed in the lungs, and a phenomenon of partial rejection can sometimes occur (see [53] for a review). But this method nevertheless proves to be useful and is often used to investigate the short to mid term effects of particles [54,55]. It is then associated with analysis of the liquid resulting from broncho-alveolar lavage (BAL) (injecting physiological solution into the lungs by the bronchial route, then recovering the liquid) to identify and quantify the cellular and humoral inflammatory response caused by the particles. The methods most recently developed for inhalation exposure favour better knowledge of the exposure dose, to the detriment of the physiological situation, using the so-called nose-only method, where the animals are immobilised and exposed in individual chambers [56]. This technique, currently widely used, may also have a biological impact due to stress [57].

All these inhalation methods have led to a way of determining the dose of particles deposited in the lungs, the clearance rate, the retention level, and in the case of fibres, the evolution of dimensional characteristics. The technique involves extracting the particles after clearance from the pulmonary tissue [58,59]. It is observed that fibres may break, and that bundles of fibres may split up to some extent, thereby altering the density of fibres of given dimensions as time goes by. Several groups used radio tagged fibres to determine the migration and bioavailability of particles in various pulmonary locations [60,61]. Many studies have shown preferential clearance of short fibres, thanks to macrophage purification, while longer fibres tend to be retained by the pulmonary parenchyma [62,63]. Note that the nose-only method sometimes returns contradictory results, a point still in need of explanation [64].

To determine the long term consequences for the serous membranes (pleura and peritoneum) of exposure to particles, methods involve intracavitary injection or implantation of particles. In the context of this historical survey, it is interesting to note that earlier work on the implantation of solid substances led to a 'solid state' theory of carcinogenesis, inspired by polymer implantation experiments [65]. This terminology was used to distinguish tumours produced by a solid agent from those produced by a chemical agent. The use of such experimental systems for particle toxicology can be related to the emergence of questions over asbestos exposure [66,67]. These methods have been criticised for their non-physiological nature. However, inhalation methods cause very little pleural reaction in rats, even with forms of asbestos that are considered to be highly carcinogenic, e.g., crocidolite, and they are not sensitive enough to assess the fibrosing and carcinogenic effect. (The life expectation of the animal is too short compared with humans, where pleural pathologies materialise only 30–40 years after the beginning of exposure.)

Over the last few years, work has been carried out using genetically modified mice and rats. These are animals in which certain genes have been deactivated (silenced), or new genes introduced by genetic manipulation into the

animals' genomes. Studies made on these animals help to get a better understanding of the mechanisms through which particles act, but also give insights into the regulatory channels that are stimulated or altered in response to the particles, identifying the factors and genes involved in the development of pathologies. Work has been done on fibrogenesis and carcinogenesis [68–73].

1.4.2 Isolated Cells

Many cell types have been used in toxicological studies of solid particles. As mentioned above, the first cytotoxicity studies were carried out with red blood cells, using hemolysis as evaluation criterion, attesting as it does to the destruction of the cell membrane by the particles. These studies were done in the context of research on silica and asbestos [74]. It is unrealistic to extrapolate the results to pathologies caused by these particles, but these studies give insights into the factors modulating cytotoxicity. They revealed an electrostatic type of interaction and adsorption of membrane phospholipids. These simple models have had interesting spin-offs as regards reflection on the interactions between a solid surface and the biological medium. They were used to show that physicochemical features, e.g., charge, redox status, surface defects, were particularly important [75–77]. They also showed that the particles were not totally inert with regard to the cells, since they had adsorption properties with respect to biological macromolecules. Current work involving nanoparticles confirms that these interactions must be taken into consideration [78].

Alveolar and peritoneal macrophages were also put to use early on, in two different approaches, either ex vivo after BAL recovery from animals exposed in vivo, or in vitro after culturing the BAL obtained from untreated animals and incubating the cells in the presence of particles. With these systems, one can study the cytotoxicity of the particles and the response of the cells, usually an inflammatory reaction, i.e., production of cytokines, growth factors, chemokines, and so on [79].

Another cell type has been used, namely the fibroblasts, exploiting their function of producing the molecules of the conjunctive tissue. These cells are used to study fibrogenesis. The response of these cells is determined either after direct exposure to particles, or in response to inflammatory factors produced by other cells exposed to particles, e.g., macrophages, epithelial cells.

Legislation to reduce dust levels in the workplace, thereby reducing the number of cases of pulmonary fibrosis, has focused concern on cancer. However, as mentioned earlier, this illness is always present in certain situations. Silica is currently used as a research tool in studies of the inflammatory mechanism and the immune reaction [80, 81]. Such observations will improve our understanding of the role played by inflammation in particle-related pulmonary pathologies. It is also due to this interest in cancer that studies on macrophages on the one hand, and epithelial cells on the other, has been pursued: the first, because they can produce factors able to interact with other

cell types, and the second, because these are the cells producing tumours in the lungs and pleura (bronchial and mesothelial cells, respectively).

Owing to difficulties in obtaining broncheal cell differentiation in cultures, these cells have been replaced by cultures of tracheal explants [82, 83]. Work is also done on 'immortalised' cells, obtained by transfer of a gene allowing these cells to divide in culture. For studies of pleural toxicology, the first cells cultured to study effects relating to pleural cancer were mesothelial pleural cells from rats [84, 85]. Human cells are also used by various groups [86, 87]. Many other types of cell, epithelial or otherwise, have been used since then to identify the effects of carcinogenic chemical molecules. Examples are bacteria (Ames test) and Chinese hamster ovary (CHO) cells, Syrian hamster embryo (SHE) cells, and mouse embryo fibroblasts (NIH3T3). For these cells, data was available about their response to carcinogenic chemicals, making it easier to interpret results obtained with particles. Depending on the cell type, different tests have been adapted to particles: mutagenesis (bacteria and mammal cells), genotoxicity (CHO, SHE), and transformation (SHE, BALB/3T3, CH310T1/2) tests. Models specifically devised to investigate the mutagenic effects of ionising radiation (A1 cells) have been applied [88]. Note that, in order to tackle the issue of mutagenic potential, the bacterial systems that proved so useful for chemical substances turned out to be less relevant for particles, since particles only have effects when internalised (phagocytosis), but they are unable to cross the bacterial wall.

Ames Test. This is a biological assay for identifying mutagenic substances. It was developed in the 1960s by B. Ames to determine the mutagenic potential of chemical substances. The idea is to examine mutations in bacteria. To do this, one uses mutant bacterial strains of *Salmonella typhimurium* which cannot grow without the availability of certain nutritive elements (more specifically, histidine), owing to a mutation on a gene regulating the use of these elements. The bacteria are incubated with the substance whose mutagenic potency is to be tested. The effect of mutagenic substances is characterised by a phenotypic reversal (reverse mutation) which allows the bacteria to grow without access to the nutritive elements needed for the growth of untreated bacteria.

Apart from assessing the genotoxic and transforming effects on target cells, work has also tried to determine the effects particles have on cell functions and specific regulatory channels (see Sect. 1.5).

1.4.3 Molecular Epidemiology

Although not a part of experimental toxicology, it is interesting to mention some methods used over the past few years to look for exposure biomarkers in cancers. In this field, studies have compared molecular characteristics of cancers in subjects exposed or not exposed to asbestos in the workplace. They have focused on the status of several genes that are important in carcinogenic mechanisms through their oncogenic role or tumour-suppressing genes: *k-RAS*, *RASSF1*, *TP53*, *EGF*, and *P16/CDKN2A*. However, there is no definitive data on the differences between subjects exposed or not exposed to asbestos [89–94].

Mutation of TP53. Several categories of genes are associated with the mechanism underlying cancerous transformation of cells. The oncogenes have increased activity in cancers as compared with their activity in normal cells. This modification occurs, for example, due to a point mutation, amplification, or translocation. In contrast, tumour-suppressing genes (TSG) are silenced in tumoral cells, e.g., by mutation or deletion. The gene *TP53* (tumor protein p53) is a TSG, coding for the protein p53. *TP53* has several functions in the regulation of cell proliferation, apoptosis, and DNA damage repair. This gene is silenced in many types of cancer. The gene *NF2* (neurofibromin 2) is another TSG, coding for the protein Nf2. This gene is well known because germinal mutations affect patients suffering from type II neurofibromatosis. This pathology is associated with benign tumours of the central nervous system (schwannomas). At the present time, few types of malignant tumour are known to silence this gene. Mesothelioma seems to be an exception since the gene *NF2* is silenced in a high proportion of cases (about 50% of cases).

1.5 Results on Particle Toxicity Mechanisms

Our understanding of the way cells work has progressed enormously due to recent advances in molecular biology and analytical tools. As a result, toxicology has turned more toward fundamental research to determine the mechanisms whereby particles act rather than work that might be more directly applicable to the problem of assessing toxicological risk. In the latter camp, research structures were rather poorly developed. This kind of research, which tries to understand mechanisms by studying cell functions or identified alterations of cells (response to stress, alterations to genetic material, to the regulation of proliferation, to the control of cell division, etc.), is opening up today to large scale global analyses of DNA and gene or protein expression. These methods are likely to develop over the coming years. The various systems used in silica- and asbestos-related research have served as a heuristic model for the study of atmospheric particles and NPs. Other systems must be imagined to improve the level of understanding and adapt to the specificities of particles. In this chapter, we shall consider only particles that have been the subject of many studies owing to the questions they raise with regard to public health.

1.5.1 Proven Major Risk Factors: Silica and Asbestos

Silica

Studies investigating the mechanisms underlying the effects of silica have shown that surface structure and physicochemical properties play a role. In the 1960s, experimental studies revealed that injections of the polymer poly-2-vinylpyridine-*N*-oxide (PVNO) could inhibit fibrogenesis produced by introducing silica into the peritoneum or the lungs [95]. This work was a continuation of other studies in which it had been observed that the toxicity of silica particles was reduced by treating them with aluminium. Assays had

even been undertaken in vivo, i.e., on living animals, to try to control the silicotic process by exposure to aluminium, and aluminium dust was considered to have a prophylactic effect against silicosis [23].

Many in vitro studies then showed that the cytotoxicity of silica was actually connected to its surface reactivity. These conclusions were reached by observing the inhibitive effect of pretreating the particles with various agents, including PVNO or proteins [75]. Using the model provided by red blood cells, the toxicity of silica could be attributed to the formation of hydrogen bonds between a donor (silicic acid formed at the particle surface) and the surface molecules of the cells, e.g., phospholipids. Subsequent work focused on surface activity.

The surface of quartz carries silanol groups (SiOH) and siloxane bridges (Si–O–Si) that get broken when water is present [96,97]. Apart from the formation of hydrogen bonds, the surface of quartz can produce reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\bullet -}$) or the hydroxyl radical ($OH^{\bullet}$) [98,99]. These species can have toxic effects, depending on the level of production and specific features of the cells, by causing peroxidation of membrane lipids, DNA damage (both nuclear and mitochondrial), and alteration of proteins and mitochondrial functions. Oxidative stress is the name for the cell's response to this agression (activation of defence channels, reduced synthesis of oxidising agents).

Later on, the surface reactivity of silica was studied in vitro, using acellular systems. It was found that ROS production depended on the surface state of the particles, which could be modified by mechanical milling, thermal treatment (heating), or chemical treatment (with acid or by adsorption) [100]. These observations confirmed the role of silanol groups, the number and availability of such groups being modified by these treatments [98,100]. According to these results, it is reasonable to suggest that the effect of silica on cells may depend on experimental conditions, since the surface state can modulate the cell response, either directly, or by influencing physiological phenomena, e.g., phagocytosis, a function which is itself ROS-producing. We thus understand why the nature of the dusts alone cannot explain their pathological effects. Their level of activity will depend on the different possible origins of the silica and the varied circumstances of the workers producing or using them.

The Red Blood Cell Model. Red blood cells are cells with no nucleus, produced by medullary erythroblasts. Their function is to fix oxygen by means of their intracellular hemoglobin and to carry that oxygen from the lungs to the body tissues. These cells were chosen to study the initial interactions between particles and the cell membrane. Lesions of the membrane can be evaluated by the release of hemoglobin into the extracellular medium.

However, the particulate mechanism is not only explained by surface reactivity. In parallel, cell cytotoxicity studies, mainly on macrophages, have demonstrated a production of reactive oxygen and reactive nitrogen species (ROS and RNS, respectively), related to phagocytosis of the particles [101, 102].

Phagocytosis begins by sequestering the particles in phagocytic vacuoles (phagosomes), into which the contents of the lysosomes are poured, associated with an acidification of the phagosomes. This may destabilise the phagosome membrane and lead to cell death.

Inflammation-related factors have been sought to understand fibrogenesis. The mechanism put forward involves phagocytosis of the silica particles by macrophages, and then, depending on the toxicity of the particles, cell death. In this case, the cell contents are released and the proteins can enter the extracellular medium, ready for further internalisation by macrophages. This cycle can continue, and it is felt that this mechanism could explain the increased autoimmune reactions observed in subjects exposed to silica [23, 80].

During silica phagocytosis without cell death, there may be macrophage activation and production of inflammatory molecules (ROS, RNS, cytokines, chemokines, growth factors), leading to neutrophil recruitment and activation of signalling channels [101, 103]. Recent studies on macrophages have helped to determine the different stages of this mechanism. The results support the assumptions about the role of phagocytic processes (phagosome destruction), by identifying the molecules involved in the cell response [80]. Note that the observed effects cannot necessarily be generalised to all cell types. In addition, several groups have demonstrated cooperation between macrophages and fibroblasts following exposure to silica particles, both in vivo and in vitro. The macrophages produce factors stimulating the proliferation of fibroblasts and collagen production [104–106].

To sum up, in certain forms, silica can produce ROS either directly or indirectly through the cell response. The latter stimulates activation of signalling channels that can cause apoptosis or the expression of genes favouring fibrosis. More recent work emphasises the role of cell–cell interactions, in particular between alveolar macrophages and epithelial cells (the role of pulmonary surfactant adsorbed at the surface of the silica particles), and the mechanisms whereby particles are recognised by the macrophages. There are receptors called scavenger receptors at the surface of the macrophages, and these bind to a wide range of ligands, including particles. Certain studies have shown, using so-called null mutants, i.e., not expressing these receptors, that apoptosis does indeed depend on the presence of these receptors. The review by Hamilton et al. [80] weighs up the strengths and weaknesses of these hypotheses about the action of silica.

Asbestos

Studies of the interactions between silica particle surfaces and cells had repercussions for investigations into the way asbestos fibres achieve their effects, and the same type of research on these fibres led to similar conclusions, revealing the role of electrical, redox, and adsorption properties of the fibre surfaces [98]. However, differences were found between chrysotile fibres and the various

kinds of amphibole. On the one hand, the surface charge and adsorption capacity are different, and on the other, the concentration of metal elements available for redox reactions is also different, including between the various kinds of amphibole. This is true in particular of the iron concentration [75].

Due to their shape, asbestos fibres have special properties. The fibrous shape, particularly of long fibres, measuring a few tens of microns, allows them to deposit themselves in the deep lung. As far as globular fibres are concerned, those with AD greater than 5 μm are retained in the upper airways and cannot reach the alveolar region. But fibres can reach these regions owing to their smaller diameters. These may vary from a few hundred nanometers down to nanometric order for chrysotile, depending on the number of elementary fibrils. As with silica, these interactions can lead to cell death or cell activation. Macrophages are not the only cells able to internalise particles. The epithelial and mesothelial cells can also do this. The result is phagocytosis of the longer fibres and a difficulty for internalisation, associated with extracellular regurgitation of intracellular factors (possible phenomenon of frustrated phagocytosis). There may also be an abnormal chromosome segregation during mitosis, as described in the literature [88, 107].

In the field of carcinogenesis, hypotheses about underlying mechanisms are based on data obtained from animal experiments and from different cell culture systems including the target cells [107, 108]. To sum up what is known about the toxicity of asbestos fibres, two non-exclusive mechanisms have been identified. One is associated with the inflammatory reaction accompanying the deposition of fibres in the airways and lungs, with a rush of inflammatory cells producing ROS, RNS, and cytokine factors. These molecules can have a genotoxic effect and favour cell proliferation. Base oxidation, in particular, of 8-hydroxy-deoxyguanosine (8-OHdG), and single-strand breaks in DNA have been detected in cells exposed to asbestos, and these might be explained by this mechanism [109, 110]. DNA damage is also suggested indirectly by the discovery that DNA repair mechanisms are activated and that the cell cycle is sometimes arrested in cells exposed to asbestos [88]. The proliferation of epithelial cells whose DNA displays damage that is either poorly repaired or not repaired at all will of course lead to an increased risk of neoplastic transformation.

Another mechanism underlying asbestos fibre toxicity, non-exclusive with regard to the last, results from the ability of epithelial and mesothelial cells to internalise the asbestos fibres. It has been shown that the phagocytosis of asbestos fibres is also associated with ROS and RNS generation, and that cell division is considerably altered by exposure to asbestos [88, 107, 109, 111]. The fibres do not seem to enter directly into the cell nuclei. However, they can end up there after mitosis, given that the nuclear membrane is destroyed during cell division and reforms within the daughter cells. Many studies on different cell types, including pleural mesothelial cells, have shown that mitosis is perturbed and chromosomes altered. In fact, various alterations have been observed, e.g., breaks in the chromosomes, abnormalities in chromosome

segregation, loss of heterozygosity [88, 112–115]. These different aberrations in the structure and number of chromosomes are not necessarily caused by mechanical effects, but may result from DNA damage or loss of control over mitosis. These effects have serious consequences for the genetic resources of the cells, in terms of both quantity of genetic material and gene expression (deletions, translocations, deregulated expression, etc.), and form part of the general mechanism of oncogenesis.

Studies carried out on animals have reproduced the pathologies observed for humans, viz., fibrosis and cancers, using different means of exposure by inhalation, intraperitoneal (IP) injection, intrapleural injection, intratracheal instillation, and intrathoracic implantation [116, 117]. A specific role played by fibre dimensions has been found in vivo in inhalation studies and intracavitary inoculation studies, as well as in culture cell experiments. In these different investigations, when comparisons are made between samples of different dimensions, it is generally observed that long fibres are more active than short ones [118]. The first work was published by Stanton et al. [119], who used intrathoracic implantation. The authors found that the highest likelihood of pleural tumours was observed for fibres of length greater than 8 µm and diameter less than 0.25 µm.

As for silica particles, the surface properties of the fibres constitute another parameter affecting their reactivity. Concerned here are the redox properties associated with the presence of metals, especially iron, playing the role of catalyst and ROS generator, adding to the ROS generated by the cells. The role played by iron turns out to be complex. Its activity depends on its oxidation state and bioavailability [75]. Adsorption of proteins like vitronectin or serum proteins on the fibre surface can modify their reactivity in cell cultures, affecting phagocytosis and ROS production. DNA can also be adsorbed onto the fibre surface. Note that asbestos fibres are efficient for transfection of genome sequences, attesting to their interaction with DNA [120–122]. The potential consequences of this property in fibre oncogenesis mechanisms have not yet been scrutinised in detail, but future studies of the interactions between DNA and nanoparticles can be expected to provide useful information in this area.

Organic molecules such as polycyclic aromatic hydrocarbons (PAH) have also been detected at the surface of these fibres, where they constitute a carcinogenic cofactor. This may explain the multiplicative effect of tobacco smoking when smokers are exposed to asbestos [123, 124].

The chemical composition of asbestos fibres also enters the equation when accounting for their carcinogenic potential, as attested by the lower tumorigenicity of chrysotile fibres when their magnesium content is reduced by acid leaching [125]. However, this same treatment modifies other fibre parameters, e.g., dimensions, surface charge, and increases the specific surface area, emphasising the importance of particle characteristics in toxicological studies.

Genuine biochemical reactions can take place between fibres and biological medium, such as the formation of asbestos bodies between asbestos fibres and cells. These formations, discovered by Marchand in 1906, comprise an asbestos

fibre core surrounded by a ferrous protein sheath [126]. This sheath seems complex, forming mainly around long fibres inside giant cells by deposition of mucopolysaccharides and calcium phosphate (apatite), and associated with a ferritin impregnation that can be converted to hemosiderin by oxidation [126].

To investigate the role of certain enzymes involved in inflammation and fibrogenesis, genetically modified mice have been used, in which a gene for modulating the inflammatory reaction has been turned off (knockout mice). The difference observed between the responses of normal and knockout subjects can be used to determine the gene's involvement in the given biological process [71, 72, 127].

Some studies have considered mutagenesis in vivo using BigBlue transgenic rats expressing the gene *lacI*. This is a way of revealing mutations, but the method has seen little development so far. An increase in the mutation rate of pulmonary DNA has been observed in BigBlue rats exposed to crocidolite by inhalation, and likewise for the DNA in peritoneal cells, after intracavitary inoculation [128, 129]. Mutations have also been detected in mice made susceptible to the development of cancers by germ cell mutation of a tumour-suppressing gene [73, 130]. Moreover, the reproduction in mice of human cancers related to a given carcinogen constitutes an interesting method for studying the mechanisms of neoplastic transformation and identifying the genes involved in oncogenesis. Knowing certain genes that are altered in human tumours, these cancers can be reproduced by a rational strategy. For example, a mutation of *TP53* has been observed in human mesothelioma in a limited number of cases, along with frequent silencing of *NF2* and genes at the locus *INK4* [131]. Exposure by intraperitoneal injection of mice that are hemizygous for a mutation of the gene *NF2* has shown that the mesotheliomas obtained with mice did indeed reproduce the characteristics of mesotheliomas described in humans. These mice were also more sensitive to mesothelioma development than non-mutant mice [130]. The mesothelioma cells obtained in this way are useful for subsequent investigation of other molecular alterations and identification of genes altered during this process.

BigBlue Rats. The genome of these rats has been modified by adding a gene *lacZ* coding for a bacterial enzyme (β-galactosidase). Each cell of these animals thus carries this gene, which serves as a tool for detecting mutations. When inserted in a cloning vector, the gene *lacZ* serves as a reporter gene on which the search for mutations will be operated. The animals are exposed to the agent under investigation, whereupon the DNA is extracted from the relevant tissues, e.g., the lungs in the case of animals that have inhaled fibres. A multistage process is then implemented to isolate the gene *lacZ* and express β-galactosidase in bacteria. The activity of this enzyme is revealed by a coloured reaction, and the β-galactosidase may or may not be functional, depending on whether the gene has mutated or not.

1.5.2 Suspected Risk Factors: Artificial Mineral Fibres

Many carcinogenicity studies on animals have focused on artificial mineral fibres (AMF), such as glass wool, rock wool, slag wool, specialty glass fibres,

and refractory ceramic fibres (RCF), with the same exposure methods as for asbestos. Inhalation studies carried out before the end of the 1980s proved negative, but the results were debated for several reasons: either because the control animals exposed to asbestos did not develop pulmonary tumours, or because the fibres used were of too high a diameter, incompatible with deposition in the lungs of the animals. Toward the end of the 1980s and the beginning of the 1990s, studies were carried out on rats and hamsters using the nose-only method. A certain number of samples (RCF) produced a significant increase in the incidence of pulmonary tumours in rats and mesotheliomas in hamsters. Exposure by intracavitary injection produced a significantly higher rate of tumours in animals treated with the fibres as compared with control animals. Recall that one of the first articles to suggest the lower toxicity of short fibres as compared with long ones was published by Stanton et al. [119], and in this study, the authors implanted 70 samples of glass fibres with various granulometric size distributions in rat pleuras.

However, this dimensional parameter could not alone explain the differences in carcinogenic potential of the various samples. Work on AMFs focused on the biodurability of these fibres, a term referring to their tendency to resist dissolving or disintegrating in the biological medium. This notion led to the idea of biopersistence which takes into account both biodurability and clearance, referring to the ability of a fibre to perdure in the lungs while conserving its chemical and physical characteristics. This in turn inspired a classification of fibre toxicity in terms of their biopersistence, the most durable fibres being considered as potentially the most carcinogenic. These studies were used to classify AMFs by the *Centre de recherche sur le cancer* (CIRC) in France and subsequently to set up a European directive (see Sect. 1.6).

One study used intratracheal instillation to investigate genotoxicity in vivo in BigBlue rats. It showed a significant increase in the mutation rate for a rock wool sample and a non-significant one for glass fibres [132].

Various cell systems have been used to study the effects of AMFs. Some samples had genotoxic effects, including DNA damage and induction of chromosomal aberrations, nuclear abnormalities, and mutations, together with a transformation of mammalian cells. In addition, fibres can cause an inflammatory reaction producing ROS, growth factors, and cytokines. ROS production by fibres does not seem to be an important characteristic of these particles.

A discussion of AMF carcinogenicity for all the different types of fibres would go beyond the scope of this review. The INSERM reports contain a discussion of the different results, while the CIRC document provides experimental details [133, 134].

1.5.3 Unknown Risk Factors: Nanoparticles

Studies on the effects of nanoparticles (NP) are flourishing. An overview of the general state of the art has been published recently [135]. There are several reviews of the latest work [46, 89, 136–138]. The experimental setups devised

for silica and asbestos studies have been applied to NPs. Widely different particles have been investigated, e.g., titanium oxide, carbon black, polystyrene, metals, metal salts, diesel smoke products, and particles from the surrounding atmosphere. Tests focused on migration and translocation, inflammatory reactions (production of inflammatory cells and factors in animals by BAL analysis), and in vitro on culture cells (inflammation, genotoxicity) [137–139].

Results showed an inflammatory response and oxidative stress in the lungs, but this response varies, and depends on the samples. The reason for these differences has not yet been identified. A lot of studies have demonstrated the genotoxic potential of NPs, but it is not yet possible to draw definitive conclusions about the parameters and factors producing these effects [140].

The penetration of NPs into cells is an important process to be taken into consideration, as for all other particles. NPs can enter cells by endocytosis, but it seems that they can also cross the cytoplasmic membrane. They may be able to enter the nucleus by transfer via the nuclear pores, or as suggested for asbestos, after mitosis [112, 140–142]. Further studies will be needed to find support for these hypotheses.

With these particles, there is some discussion over the best parameter to use for relating observed effects: mass concentration, number, surface area, and/or surface activity. The tendency is to express effects in terms of the surface area of the particles. However, a glance at the literature shows that this idea is difficult to generalise to all NPs [143]. Exposure by cutaneous NP delivery did not reveal notable effects, while systemic administration gave variable results, depending on the type of particles, characterised largely by morphological abnormalities located in the liver, the kidney, and the spleen [139]. As with asbestos and silica, knockout mice have been used, in particular to study the role of certain enzymes involved in inflammation and fibrogenesis [144].

Carbon nanotubes (CNT) have particularly interesting properties in a range of different fields of application. Many studies, including genomic methods, have demonstrated a capacity to cause oxidative stress, pulmonary inflammation, and mesotheliomas in mice. The similarity between the pathogenic properties of multiwall CNTs and asbestos fibres is currently under discussion [46, 145].

1.6 Results and Further Questions

The results of toxicological studies deserve comment in this chapter, and the historical context is relevant here. Regarding the main types of particle discussed above, the work on silica and asbestos confirms the effects on humans observed earlier on, and provides an insight into what is going on, but the exact mechanisms remain to be clarified. In the light of recent findings concerning the interactions between cells and fibres, particles cause a range of pathological consequences depending on their nature, even if they are particles with the same chemical composition as silica. Indeed, differences in

tissue and cell response are observed depending on their mineralogical nature, the surface state, and the extent of interactions between the particle surface and the cell membrane. Research in this area has also demonstrated the role played by shape and dimensions. The dependence of the effects on physical and physicochemical properties has been confirmed by studies on asbestos fibres, which justify a generalisation of these hypotheses. The incidence of the particle characteristics on biological effects is also confirmed by comparative studies of the effects of FPs and UFPs of the same chemical nature. The present understanding of the interactions between cells and silica or asbestos has influenced studies of synthetic mineral fibres, leading to the definition of biopersistence as one of the key elements determining pathological effects.

Furthermore, the data that has been built up has drawn attention to particle dynamics, and in particular, their migration toward and translocation within different organs and their chemical and dimensional evolution within the organism, avoiding the idea that they might somehow be inert as was sometimes suggested in reports on earlier observations. Bearing in mind the many applications of NPs, it is safe to predict that these questions will remain pertinent, given the tendency of NPs to aggregate and the importance of their surface properties.

In the case of asbestos, the various studies have supported epidemiological surveys, and experimental demonstrations finally led to its being outlawed in certain countries. Furthermore, studies on these minerals have stimulated research on the toxicity of other fibres, be they synthetic, inorganic, or organic, allowing us to anticipate the harmful effects resulting from these particles.

Studies carried out on silica led to certain forms being classified as carcinogenic (group 1) by the CIRC in 1996 [25]. RCFs and certain specialty glass fibres have been classified in group 2B (possibly carcinogenic for humans), whereas insulating wools have been put into group 3 (unclassifiable with regard to carcinogenicity for humans due to lack of data) [133]. Despite epidemiological studies showing various results, carbon black and titanium oxide have been classified as possibly human carcinogenic for altered clearance under high pulmonary contaminant levels, on the basis of experimental studies and effects compatible with a carcinogenic mechanism [146].

Note that, before 2006, experimental studies on animals were taken into account for this classification of carcinogenic potential, while mechanism studies carried out on isolated cells carried no weight in the final decision, but were considered only as indicators. Today studies to determine the underlying mechanisms have become a central part of CIRC assessments, while epidemiological studies are not conclusive with regard to either an absence of proof or a sufficient proof of carcinogenicity. Since 2006, mechanistic data are taken into consideration in evaluations, and can provide strong evidence for carcinogenic potential [147].

Toxicological studies of AMFs have led to the formulation of European directives for carcinogenicity tests on artificial vitrous silicate fibres. These authorise exemption from classification as ‘carcinogenic’ on the basis, for

example, of the biopersistence (half-life) of fibres in the lungs, in a short term inhalation or intratracheal instillation study [148]. Furthermore, a model has been made, using experimental data obtained with RCFs, to define an estimate of the increase in the risk of cancer associated with exposure to these fibres [149]. And hypotheses have also been formulated regarding the possible mechanisms whereby these fibres act. Using a two-stage clonal expansion model, with the stages being initiation and promotion, it has been suggested that the best fit to RCF data has the fibres as initiators [150, 151].

It may also be considered that data acquired on FPs and UFPs have stimulated interest in environmental pollution, and they have undoubtedly had consequences for investigation of the effects of NP toxicity, a subject of major importance at the present time. In addition, the protocols and methods already devised with particles in the field of inhalation toxicology will speed up investigations in other fields of exposure presently emerging with NPs, even if some adaptation will be needed. Indeed, given that these particles are present not only in aerosols, but also in other products, e.g., foods and cosmetics, other exposure routes must be taken into consideration.

These studies on particle toxicity raise a range of different questions on both the cognitive and methodological levels:

- The validity of the notion of biopersistence as an indicator of the carcinogenic potential of AMFs is still debated, and the generalisation of this notion as a means for assessing the carcinogenic potential of all fibre types has not yet been established. Note that the biopersistence of a carcinogenic agent is not a necessary factor for it to have a carcinogenic effect. Biopersistence modulates the dose rate, and introduces a time factor into the cumulative dose. Questions have been raised about the limitations of short term biopersistence studies, used to exempt AMFs from classification as carcinogens [148].
- Just as surface reactivity cannot alone explain pathological cell response, so inflammation is unable to account fully for carcinogenic effects. A recent analysis of data in the literature suggests that cancer is not necessarily related to inflammatory reaction and oxidative stress [152]. The inflammatory reaction is a natural defence process and the lungs have an antioxidant defence potential. The level of production of these reactive species must therefore be a determining factor for toxicity. Alteration of the genetic material (genetic and chromosomal mutations) is an important indicator of the carcinogenic process.
- The way particles enter into cells, and what happens to them thereafter with regard to interaction with genetic material and cell regulatory channels, need to be explored further in order to define the mechanisms of particle action and identify criteria for evaluating toxicity endpoints. To assess the potential for damage repair, it is also important to explore associated mechanisms: genetic (DNA repair), cellular (apoptosis, repopulation, etc.), and tissue (scar formation, etc.) mechanisms.

- Research carried out up to now has favoured certain mechanisms, introducing a bias toward a general understanding of cell response, focusing on one process or one mechanism. In the future, genome-wide studies should make it easier to identify regulatory channels that are activated or inhibited in cells responding to these particles, and this in a dynamic way that takes into account the microscopic surroundings of the cells.
- We should also be concerned about the best strategy or strategies to adopt to study the most representative particles in terms of risk factors, and try to identify the biological systems that are best suited to assessing hazards and risks.

1.7 Conclusions and Prospects

Particle toxicology has come into being thanks to the experimental data acquired mainly during the second half of the last century. Research brought out several mechanisms and physiological routes to be explored when examining the potential toxicity of solid particles. Points to be analysed concern not only biological aspects, such as inflammation, effects on systems regulating cell homeostasis, cell integrity, cell cooperation, and interactions between the cell and its micro-environment, but also particle aspects, i.e., physical and physicochemical characteristics. The bioavailability of the particles, their penetration into the cells, and their stability in the biological medium are important factors to take into consideration. One should expect new biological aspects and new factors to become relevant, so that new characteristics will have to be taken into account. Upstream, in order to make toxicological studies as relevant as possible, we need to ask about the context of exposure, not only with regard to the kind of particles likely to enter the organism (chemical nature, shape, and dimensions), but also with regard to the population at risk and the environmental and ecological extent of the risk. This understanding is essential for setting up the best expert systems, modelling particle–cell interactions, and determining the probability of physiopathological response. When discussing particle toxicology, the current situation is very different from previous ones, and it is essential to take this into account. Indeed, in the past, experimental studies came after the pathologies had been identified, whereas today, the new materials will precede the pathologies. Let us hope that data already made available will not simply be ignored, delaying the benefits of knowing about them when identifying and characterising new risk factors.

The significant development in the means for analysing cell functioning and the rapid expansion of means of communication make it possible today to analyse a huge volume of data, ensuring fast progress in our understanding of the life of the cell and the way it can respond to exogenous factors, in an integrated system that will take into account both biological and molecular interactions. Up to now, mechanistic studies have observed isolated responses

in a general biological and physiological context, and often under conditions that do not justify extrapolation to assess the level of risk. This integrated approach will result from large scale analyses of the various features of cell function, with benefits for toxicology and the possibility of limiting experiments on animals. Their use should be strongly encouraged to promote the rapid development of research into the effects of particles on structure, and the genetic and epigenetic modifications of genome activity. Present and future data might also be used to construct algorithms that would assist in the evaluation of toxicity, taking into account the parameters of the toxicity related to particle characteristics, biological mechanisms of the pathologies, and exposure conditions. In order to ask the relevant questions and offer efficient solutions, information must be supplied on two levels: upstream, regarding the nature of particles likely to produce health risks (role of manufacturers and safety specialists, environmental data), and downstream, regarding potential or proven risks (factory doctors, public authorities, registers, early warning systems). Considerable progress will be made in the coming years and we must acknowledge the role played by earlier investigations which, with the means available to them, laid the foundations for modern particle toxicology. Research in this area will necessarily be multidisciplinary, associating groups specialising in the physicochemical characterisation of particles and all the different aspects of biology (pathology, and cellular and molecular biology). Let us hope that the forces needed to tackle all these aspects of the research so necessary today will be successfully set in motion to achieve positive and efficient management of future health and safety requirements.

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