ZKBS
Zentrale Kommission für die Biologische Sicherheit

Ref. 402.08020203.0002.0010[1] updated July 2025

Position statement of the ZKBS on the risk assessment of genetically modified baculoviruses according to § 5 paragraph 1 GenTSV

Baculoviruses

Viruses of the family *Baculoviridae* (baculoviruses) are widespread in nature and infect the larval stages of insects of the orders *Lepidoptera* (butterflies), *Hymenoptera* (hymenopterans) and *Diptera* (dipterans) [1]. They are used as insecticides in agriculture [2] and are enveloped viruses with a double-stranded, circular DNA genome of 80 – 180 kbp. Transcription, DNA replication and nucleocapsid formation take place in the nucleus of the infected host cell. During productive infection in permissive cells, the viral DNA is not integrated into the host genome [3, 4]. The infection cycle is characterised by the formation of two different virion phenotypes: at the early stage of the replication cycle, 6 – 8 hours after infection, the budded virus (BV) is formed at the cytoplasmic membrane, and at the late stage, 18 hours after infection, the occlusion-derived virus (ODV) is formed in the cell nucleus [4]. The BV mediates cell-to-cell transmission in the infected animal and is therefore also infectious in cell culture. It enters the host cell via receptor-mediated endocytosis. The viral GP64 is a membrane fusion protein and is essential for viral budding, virus entry into the cell and cell-to-cell transmission in BV [5]. No infection occurs when BV is ingested by larval stages of the host animals, since particles do not survive the gastrointestinal passage [6]. Horizontal transmission to other host animals occurs via ODVs packaged in an envelope matrix, the occlusion bodies (OBs), which must be ingested orally. The OB contains approximately 1 to 100 ODVs. The OB's viral envelope protects the ODVs contained within its structure from external environmental influences. The predominant protein component of this protective envelope is polyhedrin (granulin in representatives of the genus *Betabaculovirus*). Polyhedrin is only formed at a very late stage of virus replication. After oral ingestion of the OB by host animals, the alkaline pH in the intestinal lumen causes the polyhedrin structure of the OB to dissolve and to release infectious ODVs. These enter the intestinal cells of the host animal through direct membrane fusion at the cell surface [1]. Successful infections with baculoviruses are lethal to host animals [7]. The viral enzymes chitinase and cathepsin, which are only expressed towards the end of the replication cycle, completely destroy the host tissue and lead to extensive liquefaction of the animal's body [8]. The liquid mass contains OBs. An exception are representatives of the genus *Betabaculovirus*, in which the infection is limited to the digestive tract and transmission occurs via the faecal-oral route [7]. Depending on the baculovirus species and influenced by the environment, infectious ODVs can survive for up to several weeks on plant parts and for months to years in soil due to the protective polyhedrin structure of the OB envelope matrix [9–11]. In

[1]former reference number 6790-05-02-41

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soil, the OBs adhere to soil particles and are no longer available for reinfection in the following vegetation period [12].

Since baculoviruses are also used as pesticides, interactions with non-target organisms have been investigated. Baculoviruses have no negative effects on solitary and social bees [13]. Furthermore, it has been shown that baculoviruses are also taken up by vertebrate cells, including human cells [2]. However, the viral genome is neither expressed nor replicated in these cells, so that no new virus particles are formed [14, 15]. In mammalian cells inoculated with the wild-type virus, no cytogenetic effects on the chromosomes of the host cell, such as chromosome aberrations and sister chromatid exchanges, were observed [16]. The inactivation of baculovirus particles in mammals is primarily facilitated by the complement system, specifically via the classical pathway, utilising the complement factor C5 [17, 18].

Baculoviruses are allocated as donor and recipient organisms for genetic engineering operations to **risk group 1** in accordance with § 5 paragraph 1 and 6 in conjunction with Annex 1 No. 1 GenTSV.

Genetically modified baculoviruses

Recombinant baculoviruses are very important for the expression of heterologous proteins in insect cells [17, 19–24]. The baculovirus most commonly used for this purpose and best characterised is *Alphabaculovirus aucalifornicae* (Autographa californica multiple nucleopolyhedrovirus, AcMNPV). The genomes of the most commonly used clones 6 and E2 have been completely sequenced and comprise approximately 133.9 kbp [25].

In the genome of recombinant AcMNPV, the polyhedrin gene (*ph* gene) is usually deleted and replaced by an expression cassette. Polyhedrin is the essential protein component of the OBs' envelope matrix [17, 18]. This envelope matrix protects the ODVs located in the OBs from external influences, enabling the infectious ODVs to survive until OB-contaminated plant parts are eaten by host animals. The OBs are also essential for passage through the digestive tract and ensure the release of ODVs in the intestine. The replication of baculoviruses, including the assembly of the capsids and the packaging of the viral DNA, takes place in the nucleus at the virogenic stroma. At an early stage of the replication cycle, when only a small quantity of genomes is packaged in capsids, these early nucleocapsids can be transported out of the nucleus, reach the cell membrane and are released there via a budding process as BVs enveloped by the cell membrane [26]. At a later stage of the replication cycle, a vast amount of nucleocapsids is formed in the nucleus so that simple transport across the nuclear membrane is no longer possible. Instead, the nucleocapsids are enveloped by the nuclear membrane. These nucleocapsids or bundles of nucleocapsids enveloped by the nuclear membrane are ODVs [26]. OBs are then formed by the inclusion of ODVs in a paracrystalline polyhedral structure [8]. When the *ph* gene is deleted, uncoated ODVs accumulate in the cell [6]. Unlike BV, ODVs are infectious to *Lepidoptera* caterpillars and remain stable at 4 °C for several days [6]. It can therefore not be ruled out that, following infection of Lepidoptera caterpillars with recombinant *ph*-deleted AcMNPV, ODVs are formed and released after liquefaction of the animal body. Recombinant *ph*-deleted AcMNPV could thus spread in the environment for short periods of time. Furthermore, it cannot be excluded that co-infection with other wild-type baculoviruses in infected caterpillars could lead to the formation of the crystalline polyhedrin envelope matrix of the recombinant AcMNPV *in trans* by the polyhedrin of the other baculovirus [27, 28].

The gene for the P10 protein is also frequently deleted in recombinant AcMNPV. The function of P10 has not yet been fully understood. It has been reported that P10 is involved in the formation of the additional polysaccharide layer of OBs, the restructuring of the cytoskeleton and the lysis of the cell nucleus and cell membrane [29, 30]. P10 is essential for cell lysis [29]. P10 is not essential for the formation of ODVs and their packaging in OBs, but deletion of the *p10* gene prevents cell lysis and OB release [31].

Recombinant AcMNPV can be taken up by mammalian cells via endocytosis or phagocytosis. The membrane fusion protein GP64 plays a crucial role for uptake. Human and primate cells efficiently take up pseudotyped viruses depending on this glycoprotein [32]. The majority of AcMNPV-specific promoters are not recognised in mammalian cells [33]. An exception is the early to late (ETL) promoter of AcMNPV, which is also active in some mammalian cells [34]. However, when the introduced transgene is under the control of expression cassettes active in mammalian cells, the heterologous genes are transiently expressed in addition to the early viral genes [17, 19–22]. A complete viral replication cycle cannot take place in the mammalian cell, consequently no viral particles are formed and released [17]. Although the introduced baculoviral DNA enters the nucleus of the transduced cell, integration only occurs under appropriate selection pressure [22, 35, 36]. Under appropriate selection pressure, illegitimate recombination can occur, in which DNA fragments ranging in size from 3.8 to 17.9 kbp can integrate into the genome at a frequency of one positive clone per 39 to 100 cells [35, 36]. Without selection pressure, the baculoviral DNA usually remains in mammalian cells for up to 14 days [37–42]. Transgene expression beyond this point is generally not detectable [43–46]. Depending on the cell type and transduction conditions, viral DNA could still be detected in differentiated myotubes after 63 days [42] and transgene expression in fish cells after 80 days [47].

Up to 38 kbp of foreign DNA can be incorporated into the recombinant AcMNPV genome with *p10* and/or *ph* deletion [48]. The expression of the transgene is then usually controlled by insect-specific promoters. To produce recombinant AcMNPV, the heterologous genes were initially inserted into the AcMNPV genome via homologous recombination. In 1993, a method was described in which the foreign gene is transferred from a donor plasmid to cloned AcMNPV DNA (bacmid) in *Escherichia coli* DH10 via specific transposition. The bacmid is a recombinant baculoviral DNA that can replicate in *E. coli* as a plasmid and is infectious for Lepidoptera cells. It contains a mini-F replicon, an antibiotic resistance marker and the target nucleic acid sequence *att*Tn7 of the Tn7 transposon. An expression cassette containing the heterologous gene under the regulatory control of the *ph* or *p10* promoter and the SV40 polyadenylation signal, flanked by the left and right ends of Tn7 and located on the donor plasmid, can be transposed in *E. coli* into the *att*Tn7 site of the bacmid when the transposition function of Tn7 is provided *in trans* by a helper plasmid. This method was called the Bac-to-Bac system [49] and was subsequently further developed [50, 51]. In order to optimise recombinant baculoviruses for gene expression in mammalian cells, vector systems were developed that simultaneously transfer two expression cassettes to AcMNPV DNA, with one cassette containing the heterologous gene of interest and the second containing the gene of a glycoprotein that is incorporated into the viral envelope for efficient transduction of mammalian cells, such as the glycoprotein of *Vesiculovirus indiana* (VSV) [52]. Another approach pursues the targeted expression of a transgene in the mammalian cell. The transgene is subject to expression control

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by an RNA polymerase II-specific promoter, such as the SV40 promoter or the *Cytomegalovirus humanbeta5* (Human cytomegalovirus, HCMV) major immediate early promoter (HCMV-MIE promoter) and the SV40 polyadenylation signal (BacMam technology) [19, 33].

In mammals baculoviral vectors can only be used *in vivo* in immune-privileged tissue types due to inactivation by the complement system. The transduction rate of baculoviral vectors when applied to the eyes of rabbits is comparable to that of vectors derived from adeno-associated viruses (AAV) [53]. As is the case with *in vitro* studies, transgene expression is detectable for a maximum of two weeks. In tissues with an active complement system, only very isolated cells are transduced, with cells near the injection site being transduced in particular [54].

In defined baculoviral vector systems, the genes *chiA* and *v-cath*, which encode chitinase and the cysteine protease cathepsin, are additionally deleted [55, 56]. The deletion of both genes prevents the vectors from liquefying animal bodies [56–58]. As a side effect, the expression of membrane-bound, nuclear, secretory and cytoplasmic proteins is improved with these vector systems [59, 60]. The virulence and formation of ODVs and OBs are not affected by the deletion of both genes [57]. Due to the absence of liquefaction of the host organism, fewer or no OBs with infectious ODVs enter the environment. This interrupts the baculoviral transmission cycle.

In addition to purely baculoviral vector systems, hybrid systems have also been developed to increase the retention time of viral DNA in vertebrate cells and extend the expression periods accordingly. For this purpose, hybrids with AAV were generated, in which a nucleic acid segment for the inverted terminal repeat was integrated into the baculoviral genome together with the *rep* reading frame of AAV to enable episomal replication or integration into human chromosome 19, where the genome of AAV vectors is preferentially integrated [44]. In other approaches, Epstein-Barr virus (EBV) hybrid vectors were generated in which the *oriP* replication origin and the *EBNA-1* gene, which controls the episomal replication of EBV, were integrated into the baculoviral genome [45]. This position statement does not cover the assessment of such hybrid vectors.

Recommendations

1) Recombinant AcMNPV with an unmodified or pseudotyped envelope that transmit a heterologous gene without risk potential are allocated to **risk group 1** in accordance with § 5 paragraph 1 in conjunction with Annex 1 No. 2 GenTSV. It is irrelevant which promoter controls the expression of the gene.
2) Recombinant AcMNPV with an unmodified or pseudotyped envelope, which heterologously expresses genes with a hazard potential for mammals, such as genes for immunomodulatory proteins (exceptions: toxins, oncogenes and prion protein genes) under the control of a promoter active in mammalian or insect cells are allocated to **risk group 1** in accordance with § 5 paragraph 1 in conjunction with Annex 1 No. 2 GenTSV.
3) Recombinant AcMNPV with an unmodified envelope encoding heterologous genes with a potential risk to insects under the control of an insect-specific promoter are allocated to **risk group 1** in accordance with § 5 paragraph 1 in conjunction with Annex 1 No. 2 GenTSV if the *chiA*, *v-cath*, *p10* and *ph* genes have been inactivated in their

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genome. If the recombinant AcMNPV are pseudotyped in such a way that the host range extends to other insect species and/or the *chiA* and *v-cath* genes are active in their genome, a case-by-case assessment is required.

4) Recombinant AcMNPV with unmodified or pseudotyped envelope, containing genes for toxins without insecticidal activity, oncogenes in accordance with the ZKBS position statement on safety measures for handling nucleic acids with neoplastic transformation potential (Ref. 6790-10-01, updated December 2016) or a prion protein (PrP) gene with deletions in the C-terminus or other PrP variants with a high probability of spontaneous conversion in accordance with the ZKBS position statement on the risk assessment of genetic engineering work for the expression of prion proteins (Ref. 402.08020203.0002.0006, updated March 2025) under the control of a promoter that is only active in insect cells (exception: highly effective toxins in accordance with § 3 sentence 5 GenTSV) are allocated to **risk group 1** in accordance with § 5 paragraph 1 in conjunction with Annex 1 No. 2 GenTSV.
5) Recombinant AcMNPV with an unmodified or pseudotyped envelope that expresses an oncogene in accordance with the ZKBS position statement on safety measures for handling nucleic acids with neoplastic transformation potential (Ref. 6790-10-01, updated December 2016) or a PrP gene with deletions in the C-terminus or other PrP variants with a high probability of spontaneous conversion in accordance with the ZKBS position statement on the risk assessment of genetic engineering work for the expression of prion proteins (Ref. 402.08020203.0002.0006, updated March 2025) in mammalian cells are allocated to **risk group 2** in accordance with § 5 paragraph 1 in conjunction with Annex 1 No. 2 GenTSV.
6) Recombinant AcMNPV with an unmodified or pseudotyped envelope carrying genes for toxins under the control of a promoter active in mammalian cells or genes for highly effective toxins under the control of a promoter active in insect cells require case-by-case evaluation.

Reasoning

Although baculoviruses are used as pesticides in agriculture and can be taken up by human and numerous animal cells, no harmful effects on humans or animals (with the exception of larvae of *Lepidoptera*, *Hymenoptera* and *Diptera*) have been identified. After pseudotyping with heterologous glycoproteins, increased efficiency of virus uptake can be assumed. However, the transferred DNA is only infectious for the cells of the corresponding insect hosts.

If the heterologous gene has no pathogenic potential, the risk potential of recombinant AcMNPV compared to wild-type baculoviruses is not increased. The viral DNA does not replicate in mammalian cells and is not integrated into the genome of the host cell without artificial selection pressure. The expression of the heterologous gene in vertebrate cells only occurs if a suitable promoter is used and is only transient (depending on the cell type for a period of one to eleven weeks).

However, if genes with neoplastic transforming potential or genes encoding PrP, which have a high probability of spontaneous conversion without physical-chemical pretreatment, are

highly expressed, a low risk to the experimenter cannot be excluded under certain circumstances. If these genes are present in cassettes that allow high expression in mammalian cells, safety level 2 measures are recommended for handling the recombinant virus particles.

For baculoviral expression of PrP in cell culture, Section 2.4 of the ZKBS position statement on the risk assessment of genetic engineering work involving the expression of prion proteins (Ref. 402.08020203.0002.0006) should be followed.

The hazard potential of baculoviral vectors that transfer genes with neoplastic transforming potential is determined by the duration and strength of expression, as well as by the effect they can generally achieve within the short time window. A downgrading of the GMO to risk group 1 is possible based on the literature and/or experimental evaluation. As an additional safety measure, the ZKBS recommends the following precautions for personal protection when handling such GMOs:

- Safety glasses should be worn
- Disposable gloves should be worn
- Mouth and nose mask to prevent nasal or oral intake through direct contact should be worn
- This work should not be carried out by people with skin barrier disorders

The transfer of any other heterologous gene with a risk potential using a recombinant AcMNPV will result in transient expression of the gene in infected insect or mammalian cells. There is generally no risk to mammals with healthy immune systems, as recombinant particles are also inactivated by the complement system and expression in single, potentially infected cells has no systemic effects and is only transient. If the *chiA*, *v-cath*, *p10* and *ph* genes in the AcMNPV genome are inactivated, the spread of such recombinant particles in *Lepidoptera*, *Hymenoptera* and *Diptera* populations can be ruled out, as no OBs are formed and the release of infectious ODVs is greatly reduced since cell lysis in the infected host cells does not occur and the animal body is not liquefied. These recombinant particles also pose no potential risk to larvae of host animals (*Lepidoptera*, *Hymenoptera* and *Diptera*).

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BVL_FO_05_4100_402_V1.9